

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 314 317 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
26.08.1988 Bulletin 1988/35

(51) Int Cl.⁶: **C12N 15/00**, C12P 21/02,
A61K 38/10
// G01N33/566

(21) Application number: 88309194.4

(22) Date of filing: 03.10.1988

(54) Adhesion variants, nucleic acid encoding them and compositions comprising them

Adhäsionsvarianten, dafür kodierende Nukleinsäure und diese enthaltende Zusammensetzungen

Variantes d'adhésions, acide nucléique les codant ainsi que compositions les contenant

(84) Designated Contracting States:
ES

(30) Priority: 02.10.1987 US 104329
28.09.1988 US 250785

(43) Date of publication of application:
03.05.1989 Bulletin 1989/18

(73) Proprietor: GENENTECH, INC.
South San Francisco California 94080 (US)

(72) Inventors:
• Capon, Daniel J.
San Mateo California 94402 (US)
• Gregory, Timothy J.
Hillsborough California 94010 (US)

(74) Representative: Armitage, Ian Michael et al
MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP (GB)

(56) References cited:
WO-A-88/01304

- CELL, vol. 42, no. 1, August 1985, pages 93-104
P.J. MADDON et al.: "The Isolation and
nucleotide sequence of a cDNA encoding the T
cell surface protein T4: a new member of the
immunoglobulin gene family"
- PROCEEDINGS OF THE NATIONAL ACADEMY
OF SCIENCES OF THE USA, vol. 84, March 1987,
pages 1649-1653 S.J. CLARK et al.: "Peptide and
nucleotide sequences of rat CD4 (W3/25)
antigen: Evidence for derivation from a structure
with four immunoglobulin-related domains"
- SCIENCE, vol. 238, December 18, 1987; pages
1704-1707; D.H. SMITH et al.: "Blocking of HIV-1
infectivity by a soluble, secreted form of the CD4
antigen"
- A.F. Williams et al., Ann. Rev. Immunol., 1988,
vol.6, pp. 381-405

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 0 314 317 B1

Description

This application relates to compositions for antiviral or immunomodulatory therapy. In particular, it relates to compositions useful in the treatment of Human Immunodeficiency Virus (HIV) infections.

The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (H. Lane *et al.*, *Ann. Rev. Immunol.* **3**:477 [1985]). CD4 is a non-polymorphic glycoprotein with homology to the immunoglobulin gene superfamily (P. Maddon *et al.*, *Cell* **42**:93 [1985]). Together with the CD8 surface antigen, CD4 defines two distinct subsets of mature peripheral T cells (E. Reinherz *et al.*, *Cell* **19**:821 [1980]), which are distinguished by their ability to interact with nominal antigen targets in the context of class I and class II major histocompatibility complex (MHC) antigens, respectively (S. Swain, *Proc. Natl. Acad. Sci.* **78**:7101 [1981]; E. Engleman *et al.*, *J. Immunol.* **127**:2124 [1981]; H. Spitz *et al.*, *J. Immunol.* **129**:1563 [1982]; W. Biddison *et al.*, *J. Exp. Med.* **156**:1065 [1982]; and D. Wilde *et al.*, *J. Immunol.* **131**:2178 [1983]). For the most part, CD4 T cells display the helper/inducer T cell phenotype (E. Reinherz, *supra*), although CD4 T cells characterized as cytotoxic/suppressor T cells have also been identified (Y. Thomas *et al.*, *J. Exp. Med.* **154**:459 [1981]; S. Meuer *et al.*, *Proc. Natl. Acad. Sci. USA* **79**:4395 [1982]; and A. Krensky *et al.*, *Proc. Natl. Acad. Sci. USA* **79**:2365 [1982]). The loss of CD4 helper/inducer T cell function probably underlies the profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of the acquired immunodeficiency syndrome (AIDS) (H. Lane *supra*).

Studies of HIV-I infection of fractionated CD4 and CD8 T cells from normal donors and AIDS patients have revealed that depletion of CD4 T cells results from the ability of HIV-I to selectively infect, replicate in, and ultimately destroy this T lymphocyte subset (D. Klatzmann *et al.*, *Science* **225**:59 [1984]). The possibility that CD4 itself is an essential component of the cellular receptor for HIV-I was first indicated by the observation that monoclonal antibodies directed against CD4 block HIV-I infection and syncytia induction (A. Dalgleish *et al.*, *Nature [London]* **312**:767 [1984]; J. McDougal *et al.*, *J. Immunol.* **135**:3151 [1985]). This hypothesis has been confirmed by the demonstration that a molecular complex forms between CD4 and gp120, the major envelope glycoprotein of HIV-I (J. McDougal *et al.*, *Science* **231**:382 [1986]; and the finding that HIV-I tropism can be conferred upon ordinarily non-permissive human cells following the stable expression of a CD4 cDNA (P. Maddon *et al.*, *Cell* **47**:333 [1986]). Furthermore, the neurotropic properties of HIV-I, reflected by a high incidence of central nervous system dysfunction in HIV-I infected individuals (W. Snider *et al.*, *Ann. Neurol.* **14**:403 [1983]), and the ability to detect HIV-I in the brain tissue and cerebrospinal fluid of AIDS patients (G. Shaw *et al.*, *Science* **227**:177 [1985]; L. Epstein, *AIDS Res.* **1**:447 [1985]; S. Koenig, *Science* **233**:1089 [1986]; D. Ho *et al.*, *N. Engl. J. Med.* **313**:1498 [1985]; J. Levy *et al.*, *Lancet* **II**:586 [1985]), appears to have its explanation in the expression of CD4 in cells of neuronal, glial and monocyte/macrophage origin (P. Maddon, *Cell* **47**:444 [1986]; I. Funke *et al.*, *J. Exp. Med.* **165**:1230 [1986]; B. Tourville *et al.*, *Science* **234**:610 [1986]).

In addition to determining the susceptibility to HIV-I infection, the manifestation of cytopathic effects in the infected host cell appears to involve CD4. Antibody to CD4 was found to inhibit the fusion of uninfected CD4 T cells with HIV-I infected cells *in vitro*; moreover, the giant multinucleated cells produced by this event die shortly after being formed resulting in the depletion of the population of CD4 cells (J. Lifson *et al.*, *Science* **232**:1123 [1986]). Formation of syncytia also requires gp120 expression, and can be elicited by coculturing CD4-positive cell lines with cell lines expressing the HIV-I *env* gene in the absence of other viral structural or regulatory proteins (J. Sodroski *et al.*, *Nature* **322**:470 [1986]; J. Lifson *et al.*, *Nature* **323**:725 [1986]). Thus, in mediating both the initial infection by HIV-I as well as eventual cell death, the interaction between gp120 and CD4 constitutes one of several critical entry points in the viral life cycle amenable to therapeutic intervention (H. Mitsuya *et al.*, *Nature* **325**:773 [1987]).

The known sequence of the CD4 precursor predicts a hydrophobic signal peptide, an extracellular region of approximately 370 amino acids, a highly hydrophobic stretch with significant identity to the membrane-spanning domain of the class II MHC beta chain, and a highly charged intracellular sequence of 40 residues (P. Madden, *Cell* **42**:93 [1985]). The extracellular domain of CD4 consists of four contiguous regions each having amino acid and structural similarity to the variable and joining (V-J) domains of immunoglobulin light chains as well as related regions in other members of the immunoglobulin gene superfamily (a subclass of which are defined herein by the coined term "adhesons". These structurally similar regions of CD4 are termed the V₁J₁, V₂J₂, V₃J₃ and V₄J₄ domains (denominated 1-4 in Fig. 3).

A successful strategy in the development of drugs for the treatment of many receptor mediated abnormalities has been the identification of antagonists which block binding of the natural ligand. Since the CD4 adheson ordinarily binds to the recognition sites of the HIV envelope it would appear to be a candidate for therapeutically sequestering these HIV sites, thereby blocking viral infectivity. However, full length CD4 and other adhesons are cell membrane proteins which are anchored in the lipid bilayer of lymphocytes. The presence of membrane components will be undesirable from the standpoint of manufacturing and purification. In addition, since adhesons are normally present only on cell surfaces, it would be desirable to produce adhesons in a form which is more stable in the circulation. Additionally, even truncated, soluble CD4 adheson (generally referred to as CD4T) may not be optimally effective as a therapeutic since

it possesses a relatively short biological half-life, binds to HIV no better than cell surface CD4, may not cross the placental or other biological barriers and since it merely sequesters the HIV recognition sites without in itself bearing an infected-cell killing or virus killing functionality.

Accordingly, it is desirable to produce soluble, secreted adhesions. It is also desirable to produce CD4 derivatives useful in the treatment of AIDS and related conditions, in a manner essentially unaffected by the extreme degree of genetic variation observed among various HIV-I isolates and their respective *env* polypeptides (J.Coffin, Cell 46:1 [1986]). It is further desirable to prepare adhesions fused to other polypeptides in order to provide molecules with novel functionalities such as those described above for therapeutic use, or diagnostic reagents for the *in vitro* assay of adhesions or their ligands. In particular, it is desirable to prepare molecules for directing toxins or effector molecules (for example the Fc domain of immunoglobulin) to cells bearing receptors for the adhesions, e.g. HIV gp120 in the case of CD4, and for use in facilitating purification of the adhesions. It is further desirable to provide stable, highly purified adhesion preparations.

The present invention provides nucleic acid encoding an amino acid sequence variant of an adhesion (CD4), in particular a variant in which the trans-membrane domain is modified so that it is no longer capable of becoming lodged in the cell membrane, i.e. inactivated, for example, by deletion or substitution.

Variant adhesions are produced by a method comprising (a) transforming a host cell with nucleic acid encoding an amino acid sequence variant of an adhesion, (b) culturing the host cell and (c) recovering the variant adhesion from the host cell culture media.

In another embodiment the invention provides an adhesion variant selected from the group consisting of (a) a CD4 adhesion amino acid sequence variant having an inactivated transmembrane domain and (b) a polypeptide comprising an adhesion extracellular domain fused to the sequence of a polypeptide which is extraneous to the adhesion, this latter for CD4 extracellular domain being for example selected from a cytotoxin, an immunogen or an immunoglobulin constant domain, and for other adhesion extracellular domains being an immunoglobulin constant region.

In a preferred embodiment a polypeptide comprising a gp120 binding domain of the CD4 adhesion is fused at its C-terminus to an immunoglobulin constant domain, or is linked to a cytotoxic polypeptide such as ricin.

The CD4 adhesion variants provided herein are purified and formulated in pharmacologically acceptable vehicles for administration to patients in need of antiviral or immunomodulatory therapy, in particular patients infected with HIV.

Brief Description of the Drawings

Figs. 1a-1c depict the amino acid and nucleotide sequence of a secreted form of the CD4 adhesion (CD4T). Other forms of CD4T terminate at Ser 366 or Pro 368. The signal processing site is designated with an arrow.

Figs. 2a-2c depict the amino acid and nucleotide sequence of a fusion of the herpes gD leader and N-terminal 27 residues to the putative mature N-terminus of CD4T.

Fig. 3 depicts the structural elements of the native and soluble CD4 antigen, the native immunoglobulin G heavy (γ) chain and two exemplary heavy chain-CD4 chimeras.

Figs. 4a-4b are a map of the linked immunoglobulin γ chain fragment employed in the preparation of CD4 fusions. Insert sites are designated $\gamma 1$ and Fc.

Fig. 5 is a map of a human light chain fragment useful for CD4 fusions at the arrow flanked by $V_k J_k$ (light variable and joining) and C_k (light constant).

Detailed Description

Adhesions are cell surface polypeptides having an extracellular domain which is of a member of the immunoglobulin gene superfamily, excluding, however, highly polymorphic members of this superfamily selected from the group of class I and class II major histocompatibility antigens, immunoglobulins and T-cell receptor α , β , γ and δ chains. Examples of adhesions include CD2, CD4, CD8, CD28, the γ , δ and ϵ chains of CD3, OX-2, Thy-1, the intercellular or neural cell adhesion molecules (I-CAM or N-CAM), neurocytoplasmic protein (NCP-3), poly-Ig receptor, myelin-associated glycoprotein (MAG), high affinity IgE receptor, the major glycoprotein of peripheral myelin (Po), platelet derived growth factor receptor, colony stimulating factor-1 receptor, macrophage Fc receptor, Fc gamma receptors and carcinoembryonic antigen. Preferred adhesions are CD4, CD8 and high affinity IgE receptor.

This invention is particularly concerned with amino acid sequence variants of adhesions. Amino acid sequence variants of adhesions are prepared with various objectives in mind, including increasing the affinity of the adhesion for its binding partner, facilitating the stability, purification and preparation of the adhesion, increasing its plasma half life, improving therapeutic efficacy as described above in the background, introducing additional functionalities and lessening the severity or occurrence of side effects during therapeutic use of the adhesion. Amino acid sequence variants of adhesions fall into one or a combination of the following classes: insertional, substitutional or deletional variants.

Insertional amino acid sequence variants are those in which one or more amino acid residues extraneous to the

adheson are introduced into a predetermined site in the adheson including the C or N termini. Such variants are referred to as fusions of the adheson and a different polypeptide are produced. Such other polypeptides contain sequences other than those which are normally found in the adheson at the inserted position. Several groups of fusions are contemplated herein. Immunologically active adheson fusions comprise an adheson and a polypeptide containing a non-adheson epitope. The non-adheson epitope is any immunologically competent polypeptide, i.e., any polypeptide which is capable of eliciting an immune response in the animal to which the fusion is to be administered or which is capable of being bound by an antibody raised against the non-adheson polypeptide. Typical non-adheson epitopes will be those which are borne by allergens, autoimmune epitopes, or other potent immunogens or antigens recognized by pre-existing antibodies in the fusion recipient, including bacterial polypeptides such as trpLE, beta-galactosidase, viral polypeptides such as herpes gD protein, and the like. Immunogenic fusions are produced by cross-linking *in vitro* or by recombinant cell culture transformed with DNA encoding an immunogenic polypeptide. It is preferable that the immunogenic fusion be one in which the immunogenic sequence is joined to or inserted into the adheson antigen or fragment thereof by a peptide bond(s). These products therefore consist of a linear polypeptide chain containing adheson epitopes and at least one epitope foreign to the adheson. It will be understood that it is within the scope of this invention to introduce the epitopes anywhere within the adheson molecule or fragment thereof. Such fusions are conveniently made in recombinant host cells or by the use of bifunctional cross-linking agents. The use of a cross-linking agent to fuse the adheson to the immunogenic polypeptide is not as desirable as a linear fusion because the cross-linked products are not as easily synthesized in structurally homogeneous form.

These immunogenic insertions are particularly useful when formulated into a pharmacologically acceptable carrier and administered to a subject in order to raise antibodies against the adheson, which antibodies in turn are useful in diagnostics or in purification of adheson by immunoaffinity techniques known *per se*. Alternatively, in the purification of adhesons, binding partners for the fused non-adheson polypeptide, e.g. antibodies, receptors or ligands, are used to adsorb the fusion from impure admixtures, after which the fusion is eluted and, if desired, the adheson is recovered from the fusion, e.g. by enzymatic cleavage.

Other fusions, which may or may not also be immunologically active, include fusions of the adheson sequence with a signal sequence heterologous to the adheson, fusions of transmembrane-modified CD4 adhesons, for example, to polypeptides having enhanced plasma half life (ordinarily >about 20 hours) such as immunoglobulin chains or fragments thereof, and fusions with cytotoxic functionalities. Signal sequence fusions are employed in order to more expeditiously direct the secretion of the adheson. The heterologous signal replaces the native adheson signal, and when the resulting fusion is recognized, i.e. processed and cleaved by the host cell, the adheson is secreted. Signals are selected based on the intended host cell, and may include bacterial yeast, mammalian and viral sequences. The herpes gD glycoprotein signal is suitable for use in mammalian expression systems.

Plasma proteins which have enhanced plasma half-life longer than that of transmembrane modified CD4 include serum albumin, immunoglobulins, apolipoproteins, and transferrin. Preferably, the adheson-plasma protein fusion is not significantly immunogenic in the animal in which it is used and the plasma protein does not cause undesirable side effects in patients by virtue of its normal biological activity.

In a specific embodiment the adheson extracellular domain is conjugated with an immunoglobulin constant region sequence. The resulting products are referred to herein as immunoadhesons. Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler *et al.*, P.N.A.S. USA 77:2197 (1980); Raso *et al.*, *Cancer Res.* 41:2073 (1981); Morrison *et al.*, *Ann. Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison *et al.*, P.N.A.S. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

Ordinarily, the domains of adhesons that are homologous to immunoglobulins and extracellular in their native environment are fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s)-like thereof, retaining at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Immunoglobulins and other polypeptides having enhanced plasma half life are fused to the extracellular or ligand binding domains of other adhesons in the same fashion.

The boundary domains for the CD4 V-like regions (V1-V4) are, respectively, about 100-109, about 175-184, about 289-298, and about 360-369 (based on the precursor CD4 amino acid sequence in which the initiating met is -25; Fig. 1a). CD4 sequences containing any of the CD4 V domains are fused to the immunoglobulin sequence. It is preferable that the V1V2 or V1V2V3V4 be fused at their C-termini to the immunoglobulin constant region. The precise site at which the fusion is made is not critical; the boundary domains noted herein are for guidance only and other sites neighboring or within the V regions may be selected in order to optimize the secretion or binding characteristics of the CD4. The optimal site will be determined by routine experimentation. In general, it has been found that the fusions are expressed intracellularly, but a great deal of variation is encountered in the degree of secretion of the fusions from

recombinant hosts. For instance, the following table demonstrates the various immunoglobulin fusions that have been obtained by the method of this invention. In all examples of CD4 immunoadhesons, the CD4 signal was used to direct secretion from 293 cells. Lower case m represents murine origin, while the lower case h designates human origin. V and C are abbreviations for immunoglobulin variable and constant domains respectively. The numerical subscripts indicate the number of parenthetical units found in the designated multimer. It will be understood that the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins. The CD4 immunoadhesons typically contained either the first N-terminal 366 residues of CD4 (CD4₄) or the first 180 N-terminal residues of CD4 (CD4₂) linked at their C-terminus to the κ (light) chain or IgG1 heavy chain constant region (γ 1).

Table I

Transfected Gene	Secreted Product
mV _{κ} C _{κ}	mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
mV _{γ1} C _{γ1}	ND
mV _{κ} C _{κ} + μ γ 1 C _{γ1}	(mV _{κ} C _{κ}) ₂ (mV _{γ1} C _{γ1}) ₂ + mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
hCD4-mC _{κ}	hCD4-mC _{κ} and/or (hCD4-mC _{κ}) ₂
hCD4-mC _{γ1}	ND
hCD4-mC _{κ} + hCD4-mC _{γ1}	(hCD4-mC _{κ}) ₂ (hCD4-mC _{γ1}) ₂ + hCD4-mC, and/or (hCD4-mC _{κ}) ₂
hCD4-hC _{κ}	hCD4-hC _{κ} and/or (hCD4-hC _{κ}) ₂
hCD4-hC _{γ1}	(hCD4-hC _{γ1}) ₂
hCD4-hC _{κ} + hCD4-hC _{γ1}	(hCD4-hC _{κ}) ₂ (hCD4-hC _{γ1}) ₂ + hCD4-hC _{κ} and/or (hCD4-hC _{κ}) ₂
mV _{κ} C _{κ} + hCD4-hC _{γ1}	(mV _{κ} C _{κ}) ₂ (hCD4-hC _{γ1}) ₂ + mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
*ND - Not detected	

It is interesting to observe from this table that the CD4-human heavy chain immunoadheson was secreted as a dimer whereas the analogous murine construction was not detected (this not excluding the intracellular accumulation of the protein, however). The ability of the hCD4-hC γ 1 transformants to produce heavy chain dimer was unexpected since previous work had suggested that immunoglobulin heavy chains are not secreted unless the hosts are cotransformed with nucleic acid encoding both heavy and light chain (Valle *et al.*, *Nature* 241:338 [1981]). According to this invention, CD4-IgG immunoadheson chimeras are readily secreted wherein the CD4 epitope is present in light chain dimers, heavy chain monomers or dimers, and heavy and light chain tetramers wherein the CD4 epitope is present fused to one or more light or heavy chains, including heterotetramers wherein up to and including all four variable region analogues are derived from CD4. Where light-heavy chain non-CD4 variable domain is present, a heterofunctional antibody thus is provided.

Various exemplary hetero- and chimeric immunoadheson antibodies produced in accordance with this invention are schematically diagrammed below. "A" means the extracellular domain of an adheson; V_L, V_H, C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin; n is an integer; and Y designates a covalent cross-linking moiety.

- (a) AC_L;
- (b) AC_L-AC_L;
- (c) AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];
- (d) AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];
- (e) AC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];
- (f) V_LC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H]; or
- (g) [A-Y]_n-[V_LC_L-V_HC_H].

The structures shown in this table show only key features, e. g. they do not show joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. These are omitted in the interests of brevity. However, where such domains are required for binding activity they shall be construed as being present in the ordinary locations which they occupy in the adhesion, immunoadhesion or immunoglobulin molecules as the case may be. These examples are representative of divalent antibodies; more complex structures would result by employing immunoglobulin heavy chain sequences from other classes, e.g. IgM. The immunoglobulin $V_L V_H$ antibody combining site also designated as the companion immunoglobulin, preferably is capable of binding to a predetermined antigen.

Suitable companion immunoglobulin combining sites and fusion partners are obtained from IgG-1, -2, -3, or 4-subtypes, IgA, IgE, IgD or IgM, but preferably IgG-1.

A preferred embodiment is a fusion of an N-terminal portion of CD4, which contains the binding site for the gp120 envelope protein of HIV, to the C-terminal F_c portion of an antibody, containing the effector functions of immunoglobulin G_1 . There are two preferred embodiments of this sort; in one, the entire heavy chain constant region is fused to a portion of CD4; in another, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG F_c chemically (residue 216, taking the first residue of heavy chain constant region to be 114 [Kabat *et al.*, "Sequences of Proteins of Immunological Interest" 4th Ed., 1987], or analogous sites of other immunoglobulins) is fused to a portion of CD4. These embodiments are described in the examples.

More particularly, those variants in which one or more immunoglobulin-like domains of an adhesion are substituted for the variable region of an immunoglobulin chain are believed to exhibit improved *in vivo* plasma half life. These chimeras are constructed in a fashion similar to chimeric antibodies in which a variable domain from a antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; Munro, Nature 312: (13 December 1984); Neuberger *et al.*, Nature 312: (13 December 1984); Sharon *et al.*, Nature 309: (24 May 1984); Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Morrison *et al.* Science 229:1202-1207 (1985); and Boulianne *et al.*, Nature 312:643-646 (13 December 1984). The DNA encoding a CD4 V-like region is cleaved by a restriction enzyme at or proximal to a DNA encoding a boundary domain and at a point at or near the DNA encoding N-terminal 5' end of the mature antigen (where use of a non-CD4 leader is contemplated) or at or proximal to the 5' end of the N-terminal coding region for the antigen (where a native CD4 signal is employed). This DNA fragment then is readily inserted into DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, tailored by deletional mutagenesis. Preferably, this is a human immunoglobulin when the variant is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams *et al.*, Biochemistry 19:2711-2719 (1980); Gough *et al.*, Biochemistry 19:2702-2710 (1980); Dolby *et al.*, P.N.A.S. USA, 77:6027-6031 (1980); Rice *et al.*, P.N.A.S. USA 79:7862-7865 (1982); Falkner *et al.*, Nature 298:286-288 (1982); and Morrison *et al.*, Ann. Rev. Immunol. 2:239-256 (1984).

DNA encoding the immunoglobulin or immunoadhesion chimeric chain(s) is transfected into a host cell for expression. If the host cell is producing a heavy chain immunoglobulin prior to transfection then one need only transfect with the adhesion fused light chain to produce a heteroantibody. Similarly, if the host cell is expressing a light chain then DNA encoding a heavy chain adhesion fusion is transfected to produce a heteroantibody. The aforementioned immunoglobulins having one or more arms bearing the adhesion domain and one or more arms bearing companion variable regions result in dual specificity for adhesion ligand and for an antigen. These are produced by the above-described recombinant methods or by *in vitro* procedures. In the latter case, for example, $F(ab')_2$ fragments of the adhesion fusion and an immunoglobulin are prepared, the $F(ab')_2$ fragments converted to Fab' fragments by reduction under mild reducing conditions, and then reoxidized in each other's presence under acidic conditions in accord with methods known *per se*. See also U.S. patent 4,444,878.

In an alternative method for producing a heterofunctional antibody, host cells producing an adhesion-immunoglobulin fusion, e.g. transfected myelomas, also are fused with B cells or hybridomas which secrete antibody having the desired companion specificity for an antigen. Heterobifunctional antibody is recovered from the culture medium of such hybridomas, and thus may be produced somewhat more conveniently than by conventional *in vitro* resorting methods (EP 68,763).

Another group of fusions are those in which an adhesion is conjugated with a toxic substance, e.g. a polypeptide such as ricin (including deglycosylated ricin A chain), diphtheria toxin A, or a non-peptidyl cytotoxin. Where the toxin is a polypeptide it is convenient to cross-link the polypeptide to the adhesion or its transmembrane-deleted variant by conventional *in vitro* protein cross-linking agents (for suitable methods for linking ricin A chain or deglycosylated A chain to CD4 see, for example, Duncan *et al.*, "Anal. Biochem." 132:68-73 [1983]; Thorpe *et al.*, "Cancer Res." 47:5924 [1987]; and Ghotie *et al.*, "Cancer Res." 48:2610 [1988]) or by recombinant synthesis as a fusion (see for example, U.S. Patent 4,765,382). Alternatively, where companion antibodies are anti-ricin antibody immunoglobulin variable domains, such immunoglobulin heteroantibodies are employed to deliver ricin to HIV infected cells following the general procedure of Raso *et al.*, Cancer Research, 41:2073 (1981).

Another class of adhesion variants are deletional variants. Deletions are characterized by the removal of one or

more amino acid residues from an adhesion sequence. Typically, the transmembrane and cytoplasmic domains of adhesions are deleted. In the case of CD4, at least residues 368 to 395 (the transmembrane region), and ordinarily 396-433 as well (the cytoplasmic domain), will be deleted to obtain secreted forms of this adhesion. Parenthetically, the amino acid residues follow the numbers given for mature CD4 as noted, for example, in figures 1a - 1c.

Substitutional variants are those in which at least one residue in the adhesion sequence has been removed and a different residue inserted in its place. The native N-terminal residue for mature CD4 is now known to be lysine. Thus, the sequence shown in Fig. 1, with an N-terminal asparagine, is an amino acid sequence variant of native mature CD4. Table 2 below describes substitutions which in general will result in fine modulation of the characteristics of the CD antigen.

TABLE 2

Original Residue	Exemplary Substitutions
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser; ala
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in adhesion properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteinyl or prolyl is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanyl, is substituted for (or by) one not having a side chain, e.g., glycyl.

A preferred class of substitutional or deletional variants are those involving the transmembrane region of the adhesion. The transmembrane region of the adhesion is a highly hydrophobic or lipophilic domain that is the proper size to span the lipid bilayer of the cellular membrane. It is believed to anchor the adhesion in the cell membrane.

Deletion or substitution of the transmembrane domain will facilitate recovery and provide a soluble form of the adhesion by reducing its cellular or membrane lipid affinity and improving its water solubility. If the transmembrane and cytoplasmic domains are deleted one avoids the introduction of potentially immunogenic epitopes, either by exposure of otherwise intracellular polypeptides that might be recognized by the body as foreign or by insertion of heterologous polypeptides that are potentially immunogenic. A principal advantage of the transmembrane deleted adhesion is that it is secreted into the culture medium of recombinant hosts. This variant is water soluble and does not have an appreciable affinity for cell membrane lipids, thus considerably simplifying its recovery from recombinant cell culture.

It will be amply apparent from the foregoing discussion that substitutions, deletions, insertions or any combination thereof are introduced to arrive at a final construct. As a general proposition, all variants will not have a functional transmembrane domain and preferably will not have a functional cytoplasmic sequence. This is generally accomplished

by deletion of the relevant domain, although adequate insertional or substitutional mutagens also can be effective for this purpose. For example, the transmembrane domain is substituted by any amino acid sequence, e.g. a random or homopolynucleic sequence of about 5 to 50 serine, threonine, lysine, arginine, glutamine, aspartic acid and like hydrophilic residues, which altogether exhibit a hydrophilic hydropathy profile, so that it is secreted into the culture medium of recombinant hosts. This variant should also be considered to be an adhesion variant.

These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the adhesion, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant adhesions also are prepared by *in vitro* synthesis. Obviously, variations made in the DNA encoding the variant adhesions must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure deleterious to expression (EP 75,444A). The CD4 variants typically exhibit the same gp120 binding activity as does the naturally-occurring prototype, although variants also are selected in order to modify the characteristics of the CD4 adhesion as indicated above.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed adhesion variants screened for the optimal combination of desired activities. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known; for example M13 primer mutagenesis.

Adhesion variants that are not capable of binding HIV gp120 are useful nonetheless as immunogens for raising antibodies to the adhesion or as immunoassay kit components (labelled, as a competitive reagent for gp120 assay, or unlabelled as a standard for an adhesion assay) so long as at least one adhesion epitope remains active.

The DNA encoding adhesions is obtained by known procedures. See Reinhardt *et al.* and Maddon *et al.*, *op cit.* In general, prokaryotes are used for cloning of CD4 variant DNA sequences. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* B, UM101 and *E. coli* χ 1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

DNA encoding the variant adhesions are inserted for expression into vectors containing promoters and control sequences which are derived from species compatible with the intended host cell. The vector ordinarily, but need not, carry a replication site as well as one or more marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using a derivative of pBR322 which is a plasmid derived from an *E. coli* species (Bolivar, *et al.*, Gene 2: 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA constructions.

Promoters suitable for use with prokaryotic hosts illustratively include the β -lactamase and lactose promoter systems (Chang *et al.*, Nature, 275: 615 [1978]; and Goeddel *et al.*, Nature 281: 544 [1979]), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res. 8: 4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the tac promoter (H. de Boer *et al.*, Proc. Natl. Acad. Sci. USA 80: 21-25 [1983]). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to DNA encoding the adhesion variant using linkers or adaptors to supply any required restriction sites (Siebenlist *et al.*, Cell 20: 269 [1980]). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antigen.

In addition to prokaryotes, eukaryotic microbes such as yeast cultures also are useful as cloning or expression hosts. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb, *et al.*, Nature 282: 39 [1979]; Kingsman *et al.*, Gene 7: 141 [1979]; Tschemper *et al.*, Gene 10: 157 [1980]) is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85: 12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective means of selection by growth in the absence of tryptophan.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem. 255: 2073 [1980]) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg. 7: 149 [1968]; and Holland, Biochemistry 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman *et al.*, European Patent Publication No. 73,657A. Yeast enhancers

also are advantageously used with yeast promoters.

Promoters are controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. the beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, **273**: 113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hind*III E restriction fragment. Greenaway, P.J. *et al.*, *Gene* **18**: 355-360 (1982). Of course, promoters from the host cell or related species also are useful herein.

DNA transcription in higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300bp, that act to increase the transcription initiation capability of a promoter. Enhancers are relatively orientation and position independent having been found 5' (Laimins, L. *et al.*, *Proc. Natl. Acad. Sci.* **78**: 993 [1981]) and 3' (Lusky, M.L., *et al.*, *Mol. Cell Bio.* **3**: 1108 [1983]) to the transcription unit, within an intron (Banerji, J.L. *et al.*, *Cell* **33**: 729 [1983]) as well as within the coding sequence itself (Osborne, T.F., *et al.*, *Mol. Cell Bio.* **4**: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the adheson.

Expression vector systems generally will contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented medium. Two examples are: CHO DHFR⁻ cells and mouse LTK⁻ cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented medium. An alternative to supplementing the medium is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, Southern P. and Berg, P., *J. Molec. Appl. Genet.* **1**: 327 (1982), mycophenolic acid, Mulligan, R.C. and Berg, P. *Science* **209**: 1422 (1980) or hygromycin, Sugden, B. *et al.*, *Mol. Cell. Biol.* **5**: 410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

"Amplification" refers to the increase or replication of an isolated region within a cell's chromosomal DNA. Amplification is achieved using a selection agent e.g. methotrexate (MTX) which inactivates DHFR. Amplification or the making of successive copies of the DHFR gene results in greater amounts of DHFR being produced in the face of greater amounts of MTX. Amplification pressure is applied notwithstanding the presence of endogenous DHFR, by adding ever greater amounts of MTX to the media. Amplification of a desired gene can be achieved by cotransfecting a mammalian host cell with a plasmid having a DNA encoding a desired protein and the DHFR or amplification gene permitting cointegration. One ensures that the cell requires more DHFR, which requirement is met by replication of the selection gene, by selecting only for cells that can grow in the presence of ever-greater MTX concentration. So long as the gene encoding a desired heterologous protein has cointegrated with the selection gene replication of this gene gives rise to replication of the gene encoding the desired protein. The result is that increased copies of the gene, i.e. an amplified gene, encoding the desired heterologous protein express more of the desired heterologous protein.

Preferred host cells for expressing the CD antigen variants of this invention are mammalian cell lines, examples including: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, Graham, F.L. *et al.* *J. Gen Virol.* **36**: 59 [1977] and 293s cells [293 subclones selected for better suspension growth]); baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)* **77**: 4216, [1980]); mouse sertoli cells (TM4, Mather, J.P., *Biol. Reprod.* **23**: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51 cells); and TRI cells (Mather, J.P. *et al.*, *Annals N.Y. Acad. Sci.*

383: 44-68 [1982]).

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. One suitable for transformation of the host cells is the method of Graham, F. and van der Eb, A., *Virology* 52: 456-457 (1973). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell walls are used as hosts, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F.N. et al., *Proc. Natl. Acad. Sci. (USA)*, 69: 2110 (1972).

Construction of suitable vectors containing the desired coding and control sequences employ standard and manipulative ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. Suitable procedures are well known for the construction described herein. See, for example, (Maniatis, T. et al., *Molecular Cloning*, 133-134 Cold Spring Harbor, [1982]; "Current Protocols in Molecular Biology", edited by Ausubel et al., [1987], pub. by Greene Publishing Associates & Wiley-Interscience).

Correct plasmid sequences are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with ligation mixtures, successful transformants selected by ampicillin or tetracycline resistance where appropriate, plasmids from the transformants prepared, and then analyzed by restriction enzyme digestion and/or sequenced by the method of Messing et al., *Nucleic Acids Res.* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Host cells are transformed with the expression vectors of this invention. Thereafter they are cultured in appropriate culture media, e.g. containing substances for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The secreted adhesion variants are recovered and purified from the culture supernatants of recombinant hosts. Typically, the supernatants are concentrated by ultrafiltration, contacted with a ligand affinity or immunoaffinity matrix so as to adsorb the adhesion variant, and eluted from the matrix. Optionally, the adhesion is purified by ion exchange chromatography.

Surprisingly, purification of soluble CD4 adhesion from culture medium was unexpectedly difficult. Notwithstanding that the hydrophobic transmembrane region of the antigen had been deleted, the antigen exhibited a strong tendency to form aggregates that could be readily removed from suspension by centrifugation at 1000 x g, and which avidly coat surfaces such as ultrafiltration membranes. This appears to result from the reduction in concentration of albumin or other serum protein (ordinarily present in the crude preparation) to a particular level, below which the truncated antigen no longer remains soluble. This phenomenon appears to be aggravated by exposure of the CD4 adhesion to low pH (< about pH 4). As a result, separation procedures (particularly those that employ acid elution, such as immunoaffinity) should be modified so that the eluate is maintained at, or immediately returned to, about neutrality. Further, a surfactant; e.g. a detergent such as Tween 80, should be included with the antigen during the separation procedure. The final purified product will be stabilized with a predetermined protein such as albumin, and/or a detergent.

The purified adhesion is formulated into conventional pharmacologically acceptable excipients.

It is administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of soluble CD4 adhesion/ml plasma. For CD4 adhesion variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma will be initially evaluated clinically in order to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor. The ordinary dosage of soluble CD4 is 100 µg/kg of patient weight/day.

The therapeutic CD4 variants are employed with other therapies and agents for the treatment of AIDS, including AZT, neutralizing antibodies and immunocytotoxins, gp120 fragments and vaccines.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on

polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally (Lawn, R. *et al.*, Nucleic Acids Res. 9: 6103-6114 [1981], and Goeddel, D. *et al.*, Nucleic Acids Res. 8: 4057 [1980]).

"Dephosphorylation" refers to the removal of the terminal 5' phosphates by treatment with bacterial alkaline phosphatase (BAP). This procedure prevents the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Procedures and reagents for dephosphorylation and other recombinant manipulations are conventional. Reactions using BAP are carried out in 50 mM Tris at 68°C to suppress the activity of any exonucleases which may be present in the enzyme preparations. Reactions were run for 1 hour. Following the reaction the DNA fragment is gel purified.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T. *et al.*, *Id.* at 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

"Filling" or "blunting" refers to the procedures by which the single stranded end in the cohesive terminus of a restriction enzyme-cleaved nucleic acid is converted to a double strand. This eliminates the cohesive terminus and forms a blunt end. This process is a versatile tool for converting a restriction cut end that may be cohesive with the ends created by only one or a few other restriction enzymes into a terminus compatible with any blunt-cutting restriction endonuclease or other filled cohesive terminus. Typically, blunting is accomplished by incubating 2-15µg of the target DNA in 10mM MgCl₂, 1mM dithiothreitol, 50mM NaCl, 10mM Tris (pH 7.5) buffer at about 37°C in the presence of 8 units of the Klenow fragment of DNA polymerase I and 250 µM of each of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. phenol and chloroform extraction and ethanol precipitation.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention. All literature citations herein are expressly incorporated by reference.

Example 1

Construction of Vectors for the Expression of Native CD4 and Secreted Derivatives

Section 1

The plasmid used for recombinant synthesis of human CD4 was pSveCD4DHFR. The plasmid was constructed as follows:

λCD4P1 containing most of the coding sequence of human CD4 (obtained from a human placental cDNA library using oligonucleotide probes based on the published sequence [Maddon *et al.* 1985]) was digested with EcoRI to produce the cDNA insert. This fragment was recovered by polyacrylamide gel electrophoresis (fragment 1).

pUC18 was digested with EcoRI and the single fragment recovered by polyacrylamide gel electrophoresis (fragment 2). Fragment 1 was ligated to fragment 2 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct DNA fragments. This plasmid is referred to as pUCCD4.

pSveE'DHFR (Muesing *et al.*, Cell 48:691-701 [1987]) was digested with KpnI and BamHI and blunted with E. coli DNA polymerase I (Klenow fragment) and the four dNTPs. Fragment 3 containing the pML-Amp^r region, SV40 early promoter, the HIV LTR, and the mouse DHFR gene was recovered by gel electrophoresis, ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the BamHI restriction site and the absence of the KpnI restriction site. This plasmid is referred to as pSveΔBKDHFR and allows EcoRI-BamHI fragments to be inserted after the SV40 early promoter and transcribed under its control, following transfection into an appropriate cell line.

Synthetic oligonucleotides (adaptors 1-8, below) were made to extend from 76 bp 5' of the initiation codon of CD4 translation to the RsaI restriction site at 121 bp 3' of the initiator, with the sequence AATT at the 5' end of the sense strand to generate an end which could ligate to an EcoRI restriction fragment. These oligonucleotides were ligated and the 204 bp fragment containing the entire sequence recovered by gel electrophoresis (fragment 4).

CD4 adaptor 1: AATTCAAGCCCAGAGCCCTGCCATTTCTGTGGGCTCAGGTCCCT
 CD4 adaptor 2: pACTGCTCAGCCCCCTTCCTCCCTCGGCAAGGCCACAATGAACCGGGGAGTC
 CD4 adaptor 3: pCCTTTTAGGCACTTGCTTCTGGTGCTGCAACTGGCGCTCCTCCCAGC
 CD4 adaptor 4: pAGCCACTCAGGGAAACAAAGTGGTGCTGGGCAAAAAAGGGGATACAGTGGAAGTACCTGT
 CD4 adaptor 5: pACAGGTCAGTTCCACTGTATCCCTTTTTTGGCCAGCACCACCTTTGTTTCC
 CD4 adaptor 6: pCTGAGTGGCTGCTGGGAGGAGCGCCAGTTGCAGCACCAGAAGCAAGT
 CD4 adaptor 7: pGCCTAAAAGGGACTCCCCGGTTCATTGTGGCCTTGCCGAGGGAGGAAGGG
 CD4 adaptor 8: GCTGAGCAGTAGGGACCTGAGCCACAGAAATGGCAGGGCTCTGGGCTTG

pUCCD4 was digested with RsaI and SstI and the 401 bp fragment containing part of the CD4 coding sequence recovered by gel electrophoresis (fragment 5). pUC18 was digested with EcoRI and SstI and the fragment comprising the bulk of the plasmid recovered by gel electrophoresis (fragment 6). Fragments 4 and 5 were ligated to fragment 6 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of the inserted synthetic DNA was checked by excising the 605 bp EcoRI-SstI fragments from several transformants and ligating them to M13mp19 which had been digested with the same enzymes. After transformation into E. coli strain JM101, single-stranded DNA was prepared and sequenced. One plasmid which contained the correct sequence was selected, and is referred to as pCD4int.

pCD4int was digested with EcoRI and SstI and fragment 7 containing the 5' end of the CD4 coding region was recovered by gel electrophoresis. pUCCD4 was digested with SstI and BamHI and the 1139 bp fragment containing the remainder of the CD4 coding region (fragment 8) recovered by gel electrophoresis.

pSveΔBKDHFR was digested with EcoRI and BamHI and fragment 9 comprising the bulk of the plasmid was isolated. Fragments 7, 8 and 9 were ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and the resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pSveCD4DHFR, and was used to direct synthesis of recombinant intact CD4.

Section 2

A plasmid was constructed to direct the synthesis of a CD4 derivative lacking the putative transmembrane domain and most of the putative cytoplasmic domain (Maddon *et al.*). This was done with the intention of creating a secreted form of CD4, based on the assumption that these domains anchor the CD4 glycoprotein to the cell membrane, and that their deletion would result in the secretion of the product. This plasmid is referred to as pSveCD4ΔN1aDHFR and was constructed as follows:

pUCCD4 was digested with SstI and TagI and the 531 bp fragment (fragment 10) recovered. pUCCD4 was digested with NlaIII and TagI and the 112 bp fragment (fragment 11) recovered. pUCCD4 was digested with BamHI and NlaIII and the 301 bp fragment (fragment 12) recovered. pCD4int was digested with SstI and BamHI and fragment 13 comprising the bulk of the plasmid recovered. Fragments 10, 11, and 12 were ligated together with fragment 13 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. Plasmid DNA from several transformants was sequenced to ensure that the 195 bp NlaIII fragment had been deleted and that the proper reading frame was restored. The resulting plasmid is referred to as pCD4ΔN1a.

pCD4ΔN1a was digested with EcoRI and BamHI and the 1541 bp fragment containing the sequence of a CD4 derivative lacking the transmembrane and cytoplasmic domains recovered (fragment 14) and ligated to fragment 9 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction

analysis for the presence of the correct fragment. This plasmid is referred to as pSVeCD4ΔN1aDHFR.

Both pSVeCD4DHFR and pSVeCD4ΔN1aDHFR were transfected into CHO cells by the same method used to establish cell lines stably expressing HIV-I polypeptides (Muesing, Smith and Capon, Cell 48:6910701 [1987]). These cells were assayed for production by radioimmunoprecipitation as described below. While no product was detected in initial experiments, subsequent experiments showed that the above described coding segment could indeed direct the synthesis of a soluble CD4 adheson variant both in CHO and 293 cells.

Section 3

A different expression system was initially used for the synthesis and expression of a CD4 variant lacking completely the cytoplasmic and transmembrane domains. The system uses the cytomegalovirus promoter and can be used in cultured cells of human origin. The first plasmid constructed for use in this system contained the entire coding region for CD4 and was intended to function as a control in the following studies. It is referred to as pRKCD4, and was constructed as follows:

pSVeCD4DHFR was digested with EcoRI and BamHI and fragment 15 containing the entire CD4 coding region was isolated. pRK5 (European Application No. 88308386.7) was digested with EcoRI and BamHI and fragment 16 comprising the bulk of the plasmid recovered by gel electrophoresis, ligated to fragment 15, and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4.

Section 4

The next plasmid constructed was designed to direct the expression of the above-mentioned (Section 3) secreted derivative of CD4. The coding region of CD4 was fused after amino acid residue 368 of mature CD4 to a sequence from pBR322 which codes for 9 more residues before a translation termination codon. This removes the putative CD4 transmembrane and cytoplasmic domains, which are presumed to anchor CD4 to the cell surface. The plasmid is referred to as pRKCD4T, and was constructed as follows:

pSVeCD4DHFR was digested with HpaII, blunted with Klenow fragment and the four dNTPs, and digested with BstEII. The 382 bp fragment (fragment 17) containing part of the CD4 coding sequence was recovered by gel electrophoresis. pSVeCD4DHFR was digested with EcoRI and BstEII and the 874 bp fragment (fragment 18) recovered. pBR322 was digested with HindIII, blunted with Klenow fragment and the four dNTPs, and digested with EcoRI. Fragment 19 comprising the bulk of the plasmid was isolated and ligated to fragments 17 and 18 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pCD4Tint.

pRK5 was digested with EcoRI and SmaI and fragment 20 comprising the bulk of the plasmid isolated. pCD4Tint was digested with EcoRI and EcoRV and the 1410 bp fragment containing the CD4 coding sequence to the HpaII site at 1176 bp 3' of the initiating codon and the 154 bp HindIII-EcoRV fragment of pBR322 was recovered (fragment 21). Fragments 20 and 21 were ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4T.

Section 5a

In order to create a secreted form of CD4 which could be purified with an antibody directed to herpes virus type I glycoprotein D, a plasmid was constructed to express a derivative of CD4T in which the region coding for the mature, processed CD4T polypeptide was fused to a sequence coding for the signal peptide and the first 27 residues of the mature type I Herpes Simplex Virus gD glycoprotein. This plasmid is referred to as pRKGD4T, and was constructed as follows:

pgDTrunc.DHFR was digested with EcoRI and PvuII and the fragment containing the coding region for the signal peptide and first 27 residues of the mature HSV I gD glycoprotein was isolated (fragment 22). pRKCD4T was digested with EcoRI and BstEII and fragment 23 containing the 3' end of the CD4 coding sequence and the pRK5 region was isolated.

Synthetic oligonucleotides GD (adaptors 1-2, below) containing the coding sequence of CD4 from the codon for the amino terminal residue of mature CD4 to the Rsa site at 121 bp 3' of translation initiation, and containing the sequence CTGCTCGAG at the 5' end of the sense strand were prepared (fragment 24). pRKCD4 was digested with RsaI and BstEII and the 665 bp fragment containing part of the coding region for CD4 was recovered (fragment 25)

and ligated to fragment 24. After digestion with BstII to ensure that only monomeric fragment was present, the 724 bp fragment containing both sequences was recovered by gel electrophoresis (fragment 26).

Fragments 22, 23 and 26 were ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of several transformants was checked to ensure that the synthetic insert was correct and that reading frame was preserved. This plasmid is referred to as pRKGD4T.

These pRK5 derived plasmids preferably were transfected into 293S cells for stable expression according to Muesing, et al. Cell 48:691 (1987) with the exception that in addition to the plasmid of interest a plasmid expressing the neomycin resistance gene pRSV neo (Gorman et al. Science 221:553-555 (1985)) was cotransfected. 293 cells also are used satisfactorily as host cells. 2 days after transfection, the cells were passaged into standard medium (1:1 F12/DME supplemented with L-glutamine, penicillin-streptomycin and 10% FBS) with 0.5 mg/ml G418 (Genticin sulfate; Gibco) for selection of stable cell lines, rather than in media containing methotrexate as shown by Muesing et al. Cells were assayed for production of CD4 or CD4 analogs by radioimmunoprecipitation. Binding studies (section 5c) used conditioned supernatants from these cells in the 1:1 F12/DME medium. Materials used in infectivity assays (section 5b) were obtained as described in section 8 below.

gD4D adaptor 1:

CTGCTCGAGCAGGGAACAAAGTGGTCTGGGCAAAAAAGGGGATACAGTGGAACTGAC

gD4D adaptor 2:

PACAGGTCAGTTCGACTGTATCCCCTTTTTTGGCCAGCACCACCTTTGTTTCCCTGCTCGA

Section 5b

The following constitutes a study of the neutralization of HIV-1 infectivity by soluble CD4 analogs. A modification of the neutralization procedure of Robert-Guroff et al., Nature 316:72 (1985) was followed. Equal volumes of inhibitor supernatant and virus (60 microliters) were incubated at 4 degrees C for 1 hour, then the same volume of H9 (Gallo et al., Science 224:500, 1984) at 5×10^6 /ml was added and incubation continued for 1 hour at 37 degrees C. Following absorption, 2.5×10^5 cells in 150 microliters were transferred to 2 ml of incubation media. After 4 days at 37 degrees C, the cultures were split 1:2 with fresh media and incubated for an additional 3 days. Cultures were harvested, reverse transcriptase activity was measured (Groopman et al., AIDS Research and Human Retroviruses 3:71, 1987), and immunofluorescence reactivity with HIV-1 positive serum was determined as described (Polesz et al., Proc. Acad. Nat. Sci. USA 77:7415, 1980). Inhibitor supernatants were obtained from confluent plate cultures of 293s/CDT4, 293s/gD4D4T cells or untransfected 293s cells by replacing the growth medium incubation media and harvesting the supernatants 24 hours later. Inhibitor supernatant replaced part or all of the incubation media during the first three days of culture as indicated in the second column of Table 3. Challenge dose of virus was 100 TCID₅₀ (Groopman et al., supra) of HIV-1 strain HTLV-III B grown in H9 cells assayed in the same system. Incubation media consisted of RPMI 1640 media containing 2mM L-glutamine, 100 units/ml penicillin, 100 micrograms/ml streptomycin, 2 micrograms/ml polybrene and 20% fetal calf serum (M.A. Bioproducts).

Table 3

Inhibitor supernatant	Dilution of Inhibitor supernatant	Indirect immunofluorescence (%) positive cells)		Reverse transcriptase (cpm/ml x 10 ⁵)	
mock-transfected	undil.; 1:4	65.3	65.5	21.8	23.9
mock-transfected	undil.; 1:4	61.2	61.1	18.5	28.1
CD4T	undil.; 1:4	0.4	18.0	0.11	5.94

Table 3 (continued)

Inhibitor supernatant	Dilution of Inhibitor supernatant	Indirect immunofluorescence (% positive cells)		Reverse transcriptase (cpm/ml x 10 ⁵)	
CD4T	undil.; 1:4	0.8	16.1	0.15	3.72
gDCD4T	undil.; 1:4	0.4	26.8	0.14	9.92
gDCD4T	undil.; 1:4	1.4	36.1	0.23	11.3

Both forms of soluble CD4 virtually abolished the growth of HIV-1, when incubated with virus-infected cells without prior dilution (Table 2). At a dilution of 1:4 the soluble CD4 preparations were only partially effective in inhibiting virus growth, however the level of fluorescent-positive cells and reverse transcriptase was still significantly lower than cultures receiving mock-transfected cell supernatants (Table 2). Since there was no significant difference in virus growth between diluted and undiluted control supernatants, nor did any of the supernatants affect the growth of uninfected H9 cells (data not shown), soluble CD4 proteins present in these supernatants were concluded to be responsible for the neutralization of HIV-1 infection of H9 cells.

Section 5c

To determine the affinity constant for interactions between gp120 and CD4 or CD4 variants, saturation binding analysis was carried out with soluble CD4 (*supra*) and detergent solubilized intact CD4 (Lasky et al. Cell 50:975 [1987]) employing radioiodinated gp120 labeled with lactoperoxidase. Binding reactions consisted of ¹²⁵I-gp120 (3 ng to 670 ng, 2.9 nCi/ng) incubated for 1 hour at 0 degrees C with cell lysates containing intact CD4 (Laskey et al., *op cit.*) or cell supernatants containing unlabeled CD4T or gDCD4T prepared as described in section 5a. Reactions (0.2ml) had a final composition of 0.5X McDougal Lysis Buffer (McDLB) (1 x McDLB contains 0.5 % Nonidet NP-40, 0.2% Na deoxycholate, 0.12 M NaCl, 0.02 M Tris-HCl, pH 8.0) and were performed in duplicate, both in the presence or absence of 50 micrograms of unlabeled purified gp120 (74 fold or greater excess). Following incubation, bound gp120 was quantitated by immunoprecipitation and counted in a gamma counter. For immunoprecipitation, binding reaction solutions were preabsorbed with 5 microliters of normal rabbit serum for one hour at 0°C, and cleared with 40 microliters of Pansorbin (10 % w/v, Calbiochem) for 30 minutes at 0 degrees C. Samples were then incubated overnight at 0 degrees C with 2 microliters of normal serum or 5 microliters (0.25 microgram) of OKT4 monoclonal antibody (Ortho) followed by collection of immune complexes with 10 microliters of Pansorbin. Precipitates were washed twice in 1X McDLB and once in water, then eluted by eluting at 100 degrees C for 2 minutes in sample buffer (0.12 M Tris-HCl pH 6.8, 4% SDS, 0.7 M mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue). CD4 molecules were bound saturably by gp120, and yielded a simple mass action binding curve. Supernatants from mock-transfected cells gave a level of specifically bound gp120 less than 1% that found for supernatants containing soluble CD4. Scatchard analysis revealed a single class of binding sites on each molecule, with apparent dissociation constants (K_d) of 1.3 x 10⁻⁹ M, 0.83 x 10⁻⁹ M and 0.72 x 10⁻⁹ M for intact CD4, CD4T and gDCD4T, respectively. The values obtained for CD4-gp120 binding in solution are comparable to the affinity previously measured for gp120 binding to CD4 on whole cells (K_d=4.0 x 10⁻⁹ M. Lasky, Cell, *supra*).

Section 6

In order to produce secreted derivatives of CD4 which are free of extraneous amino acid residues, two plasmids were constructed for expression in 293 cells. The plasmids contain CD4 genes which have been truncated without the addition of extra residues, and are referred to as pRKCD4ΔN1a and pRKCD4TP, and were constructed as follows:

Fragment 14 containing the CD4 gene with the 195 bp N1aIII restriction fragment deleted was ligated to fragment 16, which is pRK5 digested with EcoRI and BamHI. The ligation mixture was transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4ΔN1a.

Synthetic DNA was made to attach to the HpaII site at 1176bp and which when so attached would terminate translation after amino acid residue 370 of mature CD4 (fragment 27). The other end of this fragment was designed to ligate to BamHI restriction fragments. pUCCD4 was digested with BstEII and HpaII and the 382bp fragment containing part of the CD4 gene was recovered (fragment 28). Fragments 27 and 28 were ligated and then digested with BstEII to reduce dimerized fragments to monomers, and the resulting 401bp fragment was recovered (fragment 29).

pRKCD4 was digested with BstII and BamHI and the fragment comprising the bulk of the plasmid (fragment 30)

was isolated and ligated to fragment 29. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4TP. Both plasmids are transfected into 293 cells to generate stable variant CD4-expressing cell lines as described above.

Section 7

Two plasmids were constructed to direct the expression of secreted CD4 lacking extraneous amino acid residues in CHO cells. These are referred to as pSVeCD4ΔN1aSVDHFR and pSVeCD4TPSVDHFR, and were constructed as follows:

pE348HBV.E400D22 was digested with PvuI and EcoRI and the fragment containing the SV40 early promoter and part of the β-lactamase gene was recovered (fragment 31). pE348HBV.E400D22 was digested with PvuI and BamHI and the large fragment containing the balance of the β-lactamase gene as well as the SV40 early promoter and the DHFR gene was isolated (fragment 32).

Fragments 31 and 32 were ligated together with fragment 14 and transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pSVECD4ΔN1aSVDHFR. This plasmid contains the same DNA fragment encoding the soluble CD4 molecule found in the above-mentioned plasmid pSVeCD4ΔN1aDHFR (Section 2).

pRKCD4TP was digested with EcoRI and BamHI and the fragment containing the truncated CD4 coding region was isolated and ligated to fragments 31 and 32. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pSVeCD4TPSVDHFR. Both of these plasmids are transfected into CHO cells and amplified transfectants selected by methotrexate using conventional procedures.

Example 2

Fusions of the V region of the CD4 gene, which is homologous to the variable region of immunoglobulin genes (ref Maddon *et al.* 1985), to the constant (C) region of human immunoglobulin κ and γ2 chains are constructed as follows:

Synthetic DNA is made to code for the C region of human κ chain (residues 109-214) based on the sequence published by Morin *et al.*, Proc. Natl. Acad. Sci. 82:7025-7029, with the addition at the 5' end of the coding strand of the sequence GGGG, which allows this fragment to be ligated to the BspMI site at the end of the putative V-like region of CD4. At the 3' end of the coding region, a translational stop codon is added as well as a sequence which allows this end to be ligated to BamHI restriction fragments. The synthetic DNA is made in 8 fragments, 4 for each strand, 70-90 bases long. These are then allowed to anneal and ligated prior to isolation on a polyacrylamide gel (fragment 33).

pRKCD4 is digested with EcoRI and BspMI and the 478bp fragment containing the region coding for the putative V-like domain of CD4 is recovered (fragment 34). Fragments 33 and 34 are ligated together with fragment 16 (from the expression vector pRK5). The ligation mixture is transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA is prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4Ck.

A plasmid encoding a fusion of the CD4 V-like domain to the human immunoglobulin Cγ2 region is constructed in a similar fashion, and is referred to as pRKCD4Cγ2. Both of these plasmids are transfected into 293 cells, myeloma cells or other competent cells in order to obtain cell lines expressing variant CD4 molecules as described above.

Expression in CHO Cells

Plasmids were constructed to direct the expression of the immunoadhesons described above in CHO cells. These are referred to as pSVeCD4_{4γ1}SVDHFR, pSVeCD4_{2γ1}SVDHFR, pSVeCD4_{e4γ1}SVDHFR, pSVeCD4_{e2γ1}SVDHFR, pSVeCD4_{4κ}SVDHFR and pSVeCD4_{2κ}SVDHFR.

Fragment 31 was prepared as described above. Fragment 32a was prepared by digesting plasmid pE348HBV.E400 D22 with BamHI, blunting with Klenow fragment and the four dNTPs, then digesting with PvuI. Plasmids pRKCD4_{4γ1}, pRKCD4_{2γ1}, pRKCD4_{e4γ1}, pRKCD4_{e2γ1}, pRKCD4_{4κ} and pRKCD4_{2κ} were separately digested with HindIII, blunted with Klenow fragment and the four dNTPs, then digested with EcoRI. The resulting DNA fragments were ligated together with fragments 31 and 32a and transformed into *E. coli* strain 294. Colonies were selected and checked for the presence of the correct plasmid as above, then transfected into CHO cells and amplified by methotrexate selection using conventional procedures.

Example 3

The gDCD4T secreted by the method of Example 1 was purified from cell culture fluid containing either 10% FBS (fetal bovine serum) or no added FBS. The conditioned cell culture fluid was first concentrated by ultrafiltration then purified by immunoaffinity chromatography. The immunoaffinity column was produced by coupling murine monoclonal antibody 5B6 (whose epitope is on the HSV-1 gD portion of the gDCD4T molecule) to glyceryl coated controlled pore glass by the method of Roy *et al.* 1984. The concentrated cell culture fluid is applied directly to the column and the contaminating proteins are washed away with neutral pH buffer. The column is then washed with neutral buffer containing tetramethylammonium chloride followed by neutral buffer containing Tween 80. The bound gDCD4T is eluted from the column with buffer at pH3 containing Tween 80 (0.1% w/v) and is neutralized immediately as it is eluted. The eluted neutralized gDCD4T is then concentrated by ultrafiltration and dialyzed/diafiltered to exchange the buffer for a physiological salt solution containing Tween 80 at approximately 0.1% w/v.

If the detergent is not present the gDCD4T forms aggregates as evidenced by the ability of centrifugation at approximately 10,000 Xg for 2 minutes to remove the gDCD4T from the solution. Incubation of gDCD4T at 4°C in 0.1M sodium acetate, 0.5M NaCl and 0.25M tris at pH 7 together with BSA, Tween 80 or glycerol as candidate stabilizers showed that, in the absence of a stabilizer the gDCD4T gradually aggregated over the space of 12 days to the point where only about 60-70% of the protein was soluble. However, use of 0.1% w/v Tween 80 or (0.5 mg/ml BSA ensured that about 100% or 80%, respectively, of the gDCD4T remained soluble over this period. Surprisingly glycerol was ineffective as a stabilizer and produced results inferior even to the control-at 8 days about 80% of the gDCD4T was aggregated when stored in the presence of glycerol.

Example 4

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin $\gamma 1$. These plasmids are referred to as pRKCD4_{2 γ 1}, pRKCD4_{4 γ 1}, pRKCD4_{2 γ 1}, pRKCD4_{2 γ 1}, pRKCD4_{1 γ 1}, and pRKCD4_{1 γ 1}.

Plasmid pRKCD4_{4 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

Plasmid pRKCD4_{2 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

Plasmid pRKCD4_{2 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for glutamine residue 180 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

Plasmid pRKCD4_{2 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 177 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

Plasmid pRKCD4_{1 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for aspartic acid residue 105 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature immunoglobulin $\gamma 1$ (Kabat *et al.*).

Plasmid pRKCD4_{1 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 100 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

Construction of these plasmids required the prior construction of plasmid pRKCD4TP/ $\gamma 1$. It was constructed as follows:

A cDNA clone coding for human immunoglobulin $\gamma 1$ was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Ellison *et al.*, "Nucl. Acids Res." 10: 4071-4079 [1982]), and an EcoRI-EagI fragment (the EcoRI site was contributed by a linker) containing part of the variable and all of the constant region was obtained. This fragment was blunted with Klenow fragment, and recovered by gel electrophoresis (Fragment al).

Plasmid pRKCD4TP was digested with XbaI and treated with Klenow Enzyme, and Fragment a2, containing the

linearized plasmid was recovered by gel electrophoresis, and ligated with fragment α l. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from the transformants and checked by restriction analysis for the presence of the correct fragment in the correct orientation (i.e., the immunoglobulin coding region in the same orientation as the CD4 coding region, and at the 3' end of the CD4 coding region). This plasmid is referred to as pRKCD4TP/ γ 1.

Synthetic oligonucleotides were made as primers for deletional mutagenesis reactions to fuse the appropriate coding sequences of IgG1 and CD4 as described above. These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH₂-terminal 8 residues of the desired immunoglobulin moiety). Plasmid pRKCD4TP/ γ 1 was transformed into *E. coli* strain SR101 and the transformed cultures plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13KO7 bacteriophage to yield secreted, encapsidated single-stranded templates of pRKCD4TP/ γ 1. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed *E. coli* SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (ref. Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13KO7 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmids were transfected into 293 cells using standard procedures and assayed for expression and production as described above.

	Expressed	Secreted
pRKCD4 _{e1} γ 1	-	-
pRKCD4 ₁ γ 1	+	-
pRKCD4 _{e2} γ 1	+	+
pRKCD4 ₂ γ 1	+	+
pRKCD4 _{e4} γ 1	+	+
pRKCD4 ₄ γ 1	+	+

Plasmids also were constructed to direct the expression of fusion proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the truncated portion of the constant region of human immunoglobulin γ 1, comprising only the hinge region and constant domains CH₂ and CH₃.

Synthetic oligonucleotides were made as primers for mutagenesis reactions to delete the immunoglobulin sequence from Ser114 to Cys215 inclusive (Kabat *et al.*). These were synthesized as 48-mers comprising 24 γ nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH₂-terminal 8 residues of the desired immunoglobulin moiety). Plasmids pRKCD4₄ γ 1, pRKCD4₂ γ 1 and pRKCD4₁ γ 1 were separately transformed into *E. coli* strain SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13KO7 bacteriophage. Templates were prepared as above and screened by sequencing.

The plasmid derived from plasmid pRKCD4₄ γ 1 is referred to as pRKCD4_{4Fc1}, that derived from plasmid pRKCD4₂ γ 1 is referred to as pRKCD4_{2Fc1} and that derived from plasmid pRKCD4₁ γ 1 is referred to as pRKCD4_{1Fc1}.

pRKCD4_{2Fc1}, pRKCD4_{1Fc1} and pRKCD4_{4Fc1} are cultured in the same fashion as described above and CH1-deleted CD4 immunoadhesions recovered as described elsewhere herein.

Light Chain Fusions

Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin κ . These plasmids are referred to as pRKCD4₄ κ , and pRKCD4_{e4} κ .

Plasmid pRKCD4₄ κ contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon

for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin κ , starting at the codon for threonine residue 109 of the mature human immunoglobulin κ . (Kabat *et al.*)

Plasmid pRKCD4_{e4 κ} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin κ , starting at the codon for threonine residue 109 of the mature human immunoglobulin κ (Kabat *et al.*)

These plasmids were constructed in a manner analogous to plasmid pRKCD4₁ described above, with the following exception:

The human immunoglobulin κ coding sequence (Fig. 5) was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Hieter, P.A. *et al.*, Cell 22:197-207 [1980]) and an *EcoRI*-*Bsp*MI fragment containing part of the variable region and the entire constant region was obtained. This fragment was blunted with Klenow fragment and the four dNTPs. This fragment was used instead of fragment a1, and was used to construct plasmid pRKCD4TP/h κ .

Example 5

Culture, Purification and formulation of CD4 variants

Plasmids encoding CD4T, prolyl terminal (CD4TP), or CD4T immunoadhesons were calcium phosphate transfected into CHO-DP7 (a proinsulin-transformed autocrine host cell derived from CHO; U.S.S.N. 97,472) and the transformants grown in selective medium (1:1 HAM F12/DMEM GHT⁻ containing 1 - 10% diafiltered or dialyzed bovine serum). Other suitable host cells are CHO cells or 293s human embryonic kidney cells. The transformants were sub-cloned into the same medium but containing 500 nm methotrexate. A subclone capable of secreting CD4T, CD4tp 500 b, was selected. CD4tp 500 b is cultured in a DMEM/HAM F12 medium at about 37°C until CD4T accumulates in the culture, after which the medium is separated from the cells and insoluble matter by centrifuging.

Culture fluid from CD4TP transformants was concentrated and diafiltered to lower the ionic strength. The concentrate was passed through a large volume of Q-Sepharose anion exchange resin (previously equilibrated with 25 mM NaCl, pH 8.5) in order to adsorb contaminants from the culture fluid. The isoelectric point of CD4TP is about 9.5, thus making it possible to discriminate between truncated forms of CD4 and most contaminants by alternate adsorption, respectively, on a cation exchange resin such as carboxymethyl or sulfonyl Sepharose, and an anion exchange resin such as a quaternary ammonium Sepharose. In addition, since highly electropositive domains are present in the extracellular segment of CD4 any CD4-containing variant is purified in the same fashion as CD4TP. The unadsorbed culture fluid from the anion exchange resin step was then passed through a cation exchange resin (previously equilibrated with 25 mM NaCl at pH 8.5) whereby CD4TP was adsorbed to the resin. The CD4TP was eluted with a NaCl gradient at pH 8.5, this CD variant eluting at about 0.2 mM NaCl. Ammonium sulfate was added to the eluate to the concentration of 1.7M and the solution passed through a column of hydrophobic interaction chromatography resin (phenyl or butyl Sepharose). The CD4TP was eluted from the hydrophobic interaction column with a gradient of ammonium sulfate, the CD4TP emerging at about 0.7M ammonium sulfate. The eluate was concentrated and buffer exchanged on a G-25 column using phosphate buffered saline containing .02 % (w/v) Tween 20 or Tween 80. The CD4TP was soluble and stable in this solution, which was sterile filtered and filled into vials as an aqueous formulation. Other polymeric nonionic surfactants are suitably used with the CD4 formulations, including Pluronic block copolymers or polyethylene glycol.

It is also possible to employ immunoaffinity purification of soluble CD4 wherein the CD4 is adsorbed onto an immobilized antibody against CD4. This method suffers from the disadvantage that elution of the CD4T under acidic conditions leads to protein aggregation that is only thoroughly ameliorated at relatively higher levels of surfactant. The foregoing procedure permits the use of much lower quantities of surfactant, about from 0.01 to 0.10 % (w/v) surfactant.

The procedure followed for the purification of CD4 fusions with immunoglobulin heavy chain was to concentrate recombinant supernatants by ultrafiltration and thereafter adsorb the fusion onto resin-immobilized Staphylococcal protein A. The fusion was eluted with 0.1M citrate buffer pH 3 with no salt or detergent. This preparation is buffered into tris buffer at pH 7.5. The immunoglobulin fusions with CD4 V1-V4 optionally are further purified by the procedure described above for unfused CD4 variants. CD4 immunoglobulin fusions with CD4 V1-V2 also may be purified by the procedure above, except that it is not expected that the isoelectric point of this class of molecules will be as alkaline as that of species containing all four V regions of CD4.

Example 6

The characteristics of several adhesion variants were determined. As shown in table 4 the immunoadhesons CD4₄₁₁

and CD4_{2γ1} show improved plasma half-life in rabbits, coupled with high-affinity gp120 binding and an affinity for FCγ receptor (determined with U937 cells) that is comparable to that of bulk human IgG.

Table 4

	gp120 KD (nM) [#]	FCγR KD (nM) ⁺	Plasma Half-Life ⁺⁺ In Rabbits (Hrs.)
CD4T§	2.3 ± 0.4	-	0.25
CD4 _{4γ1}	1.2 ± 0.1	2.83 ± 0.25	6.4
CD4 _{2γ1}	1.4 ± 0.1	3.01 ± 0.68	40.6
human IgG	ND	3.52 ± 0.5	21 days*

* determined in humans

⁺ KD was determined by the method of Anderson *et al.*, "J. Immunol." 125:2735-2741 (1980).

[#] determined by the method of Smith *et al.*, "Science" 238:1704-07 (1987).

§ residues 1-368 only

⁺⁺ The adhesion variant was injected intravenously into rabbits and samples of blood were collected periodically and assayed for the presence of the adhesion variant.

Claims

1. A process which comprises making a polypeptide comprising the extracellular domain sequence of a cell surface member of the immunoglobulin gene superfamily, which superfamily member is not a class I or class II major histocompatibility antigen, an immunoglobulin, nor a T-cell receptor α, β, γ or δ chain, fused to an immunoglobulin constant region.

2. The process of claim 1 wherein the polypeptide is

(a) AC_L;

(b) AC_L-AC_L;

(c) AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(d) AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(e) AC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(f) V_LC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(g) [A-Y]_n-[V_LC_L-V_HC_H]₂

wherein A is a said extracellular domain; V_L, V_H, C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin; n is an integer; and Y designates the residue of a covalent cross-linking agent.

3. The process of claim 2 wherein the V_L and V_H domains are capable of binding a predetermined antigen.

4. The process of any preceding claim wherein the immunoglobulin sequence is obtained from IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.

5. The process of any preceding claim wherein the superfamily member is CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, an intercellular or neural adhesion molecule, poly-Ig receptor, myelin associated glycoprotein, high affinity IgE receptor, platelet derived growth factor receptor, colony stimulating factor-1 receptor, Fc gamma receptors or the carcinoembryonic antigen.

6. The process of claim 5 wherein the superfamily member is CD2, CD4, CD8, CD28, the γ, δ or ε chain of CD3 or the high affinity IgE receptor.

7. The process of claim 6 wherein the superfamily member is CD4.

8. The process of any preceding claim wherein in the polypeptide said extracellular domain is fused at its C-terminus to the N-terminus of said constant region.

9. The process of claim 8 wherein the V₁V₂ or V₁V₂V₃V₄ domains of CD4 are fused at their C-termini to said constant

region.

10. The process of any preceding claim wherein the immunoglobulin constant region is of an immunoglobulin heavy chain.
11. The process of any of claims 1 to 9 wherein the immunoglobulin constant region is of an immunoglobulin light chain.
12. A process which comprises making a polypeptide comprising an extracellular domain of CD4 fused to a polypeptide extraneous to CD4.
13. The process of claim 12 wherein the extraneous polypeptide comprises a signal sequence.
14. The process of claim 12 or claim 13 wherein the extraneous polypeptide is capable of eliciting a humoral immune response in an animal.
15. The process of claim 14 wherein the extraneous polypeptide is a viral polypeptide or an allergen.
16. The process of claim 12 wherein the extraneous polypeptide is albumin, apolipoprotein or transferrin.
17. The process of claim 12 wherein the extraneous polypeptide is a cytotoxic polypeptide.
18. A process which comprises making a CD4 polypeptide amino acid sequence variant (a) having a transmembrane domain modified to be incapable of becoming lodged in the cell membrane, or (b) lacking a transmembrane domain.
19. A process according to claim 18 wherein the transmembrane domain has been modified by substituting an amino acid sequence having a substantially hydrophilic hydropathy profile.
20. A process which comprises making a composition comprising a CD4 polypeptide amino acid sequence variant (a) having a transmembrane domain modified to be incapable of cell membrane anchorage, or (b) lacking a transmembrane domain.
21. The process of claim 20 wherein the variant comprises a CD4 amino acid sequence capable of binding gp120.
22. A process which comprises making a CD4 polypeptide variant comprising a fusion protein in which a polypeptide comprising a human CD4 antigen variable (V) region is fused at its C-terminus to the N-terminus of a polypeptide comprising a constant region of an immunoglobulin chain covalently associated with a companion immunoglobulin heavy chain-light chain pair bearing a $V_L V_H$ antibody combining site capable of binding to a predetermined antigen.
23. The process of claim 22 wherein the CD4 polypeptide variant is $AC_H-(V_L C_L-V_H C_H)$, $(AC_L-AC_H)-(V_L C_L-V_H C_H)$, $(AC_L-V_H C_H)-(V_L C_L-V_H C_H)$, or $(V_L C_L-AC_H)-(V_L C_L-V_H C_H)$, wherein A is a human CD4 antigen V region, V_L and V_H are the variable domains of an immunoglobulin light and heavy chain, respectively, and C_L and C_H are the constant domains of an immunoglobulin light and heavy chain, respectively.
24. The process of claim 22 or claim 23 wherein the fusion protein comprises the V-J domain of the human CD4 antigen.
25. The process of claim 22 or claim 23 wherein the human CD4 antigen comprises the sequence Asn-Lys-Val-Val-Leu-Gly-Lys-Lys.
26. The process of any one of claims 22 to 25 wherein the immunoglobulin sequences are from IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.
27. The process of claim 26 wherein the immunoglobulin sequences are from IgG-1 or IgG-3.
28. The process of claim 26 wherein the immunoglobulin sequences are from IgA or IgM.
29. A process which comprises making nucleic acid encoding a polypeptide according to any one of claims 1 to 11.
30. A process which comprises making replicable vector comprising nucleic acid prepared by the process of claim 29.

31. A recombinant host cell comprising nucleic acid prepared by the process of claim 29 or the recombinant vector prepared by the process of claim 30.
32. A method for preparing a polypeptide according to any one of claims 1 to 11 comprising recovering the polypeptide from the culture of a recombinant host cell according to claim 31.
33. A process which comprises making nucleic acid encoding a polypeptide according to any one of claims 12 to 17.
34. A process which comprises making a replicable vector comprising nucleic acid prepared by the process of claim 33.
35. A recombinant host cell comprising nucleic acid prepared by the process of claim 33 or the recombinant vector prepared by the process of claim 34.
36. A method for preparing a polypeptide according to any one of claims 12 to 17 comprising recovering the polypeptide from the culture of a recombinant host cell according to claim 35.
37. A process which comprises making nucleic acid encoding a polypeptide according to claim 18 or claim 19.
38. A method for preparing a CD4 polypeptide amino acid sequence variant according to claim 18 or claim 19 comprising recovering the variant from the culture of a host cell transfected with the nucleic acid prepared by the process of claim 37.
39. A process which comprises making nucleic acid encoding a CD4 polypeptide variant according to any one of claims 22 to 29.
40. A method of preparing a CD4 polypeptide variant comprising transfecting a host cell expressing nucleic acid encoding an immunoglobulin having a variable region directed against a predetermined antigen, with nucleic acid encoding a fusion protein in which a polypeptide comprising a human CD4 antigen variable (V) region is fused at its C-terminus to the N-terminus of a polypeptide comprising a constant region of an immunoglobulin chain.
41. The method of claim 40 wherein the CD4 polypeptide variant is recovered from the host cell culture.

Patentansprüche

1. Verfahren zur Herstellung eines Polypeptids, umfassend die Sequenz der extrazellulären Domäne eines Zelloberflächenmitglieds der Immunglobulin-Gen-Überfamilie, welches Überfamilienmitglied kein Haupt-Histokompatibilitäts-Antigen der Klasse I oder Klasse II, kein Immunglobulin und weder die α -, β -, γ - noch die δ -Kette eines T-Zellenrezeptors ist, fusioniert mit einem konstanten Immunglobulin-Bereich.
2. Verfahren nach Anspruch 1, worin das Polypeptid

- (a) AC_L ;
- (b) AC_L-AC_L ;
- (c) $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ oder } V_LC_L-V_HC_H]$;
- (d) $AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ oder } V_LC_L-V_HC_H]$;
- (e) $AC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ oder } V_LC_L-V_HC_H]$;
- (f) $V_LC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ oder } V_LC_L-V_HC_H]$;
- (g) $[A-Y]_n-[V_LC_L-V_HC_H]_2$

ist, worin A die extrazelluläre Domäne ist, V_L , V_H , C_L und C_H variable bzw. konstante Leicht- bzw. Schwereketten-Domänen eines Immunglobulins darstellen; n eine ganze Zahl ist; und Y den Rest eines kovalenten Vernetzers bezeichnet.

3. Verfahren nach Anspruch 2, worin V_L - und V_H -Domänen zur Bindung eines vorbestimmten Antigens fähig sind.
4. Verfahren nach einem der vorangegangenen Ansprüche, worin die Immunglobulinsequenz von IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD oder IgM stammt.

5. Verfahren nach einem der vorangegangenen Ansprüche, worin das Überfamilienmitglied CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, ein interzelluläres oder neurales Bindemolekül, Poly-Ig-Rezeptor, mit Myelin assoziiertes Glykoprotein, IgE-Rezeptor mit hoher Affinität, von Blutplättchen stammender Wachstumsfaktorrezeptor, Kolonie stimulierender Faktor-1-Rezeptor, Fc- γ -Rezeptoren oder das carcinoembryonale Antigen ist.
6. Verfahren nach Anspruch 5, worin das Überfamilienmitglied CD2, CD4, CD8, CD28, die γ -, δ - oder ϵ -Kette von CD3 oder der IgE-Rezeptor mit hoher Affinität ist.
7. Verfahren nach Anspruch 6, worin das Überfamilienmitglied CD4 ist.
8. Verfahren nach einem der vorangegangenen Ansprüche, worin die extrazelluläre Domäne im Polypeptid an ihrem C-Terminus mit dem N-Terminus des konstanten Bereichs fusioniert ist.
9. Verfahren nach Anspruch 8, worin die V_1V_2 - oder $V_1V_2V_3V_4$ -Domänen von CD4 an ihren C-Termini mit dem konstanten Bereich fusioniert sind.
10. Verfahren nach einem der vorangegangenen Ansprüche, worin der konstante Immunglobulinbereich von der schweren Kette eines Immunglobulins stammt.
11. Verfahren nach einem der Ansprüche 1 bis 9, worin der konstante Immunglobulinbereich von der leichten Kette eines Immunglobulins stammt.
12. Verfahren zur Herstellung eines Polypeptids, umfassend eine extrazelluläre Domäne von CD4, fusioniert mit einem zu CD4 fremden Polypeptid.
13. Verfahren nach Anspruch 12, worin das fremde Polypeptid eine Signalsequenz umfaßt.
14. Verfahren nach Anspruch 12 oder 13, worin das fremde Polypeptid bei einem Tier eine humorale Immunreaktion hervorrufen kann.
15. Verfahren nach Anspruch 14, worin das fremde Polypeptid ein virales Polypeptid oder ein Allergen ist.
16. Verfahren nach Anspruch 12, worin das fremde Polypeptid Albumin, Apolipoprotein oder Transferrin ist.
17. Verfahren nach Anspruch 12, worin das fremde Polypeptid ein zytotoxisches Polypeptid ist.
18. Verfahren nach Anspruch 12, worin die Transmembrandomäne so modifiziert ist, daß sie nicht mehr fähig ist, sich in der Zellmembran anzuordnen, oder der (b) eine Transmembrandomäne fehlt.
19. Verfahren nach Anspruch 18, worin die Transmembrandomäne durch Substitution einer Aminosäuresequenz mit einem im wesentlichen hydrophilen Hydropathieprofil modifiziert worden ist.
20. Verfahren zur Herstellung einer Zusammensetzung, umfassend eine CD4-Polypeptid-Aminosäuresequenzvariante, die (a) eine Transmembrandomäne aufweist, die so modifiziert ist, daß sie nicht mehr zur Verankerung in der Zellmembran fähig ist, oder der (b) eine Transmembrandomäne fehlt.
21. Verfahren nach Anspruch 20, worin die Variante eine CD4-Aminosäuresequenz umfaßt, die zur Bindung von gp120 fähig ist.
22. Verfahren zur Herstellung einer CD4-Polypeptidvariante, die ein Fusionsprotein umfaßt, in dem ein Polypeptid, das einen variablen (V-) menschlichen CD4-Antigen-Bereich umfaßt, an seinem C-Terminus mit dem N-Terminus eines Polypeptids fusioniert ist, das einen konstanten Bereich einer Immunglobulinkette umfaßt, die kovalent mit einem begleitenden Immunglobulin-Schwerketten-Leichtketten-Paar assoziiert ist, das eine V_LV_H -Antikörperkombinationsstelle umfaßt, die zur Bindung an ein vorbestimmtes Antigen fähig ist.
23. Verfahren nach Anspruch 22, worin die CD4-Polypeptidvariante $AC_H(V_LC_L-V_HC_H)$, $(AC_L-AC_H)-(V_LC_L-V_HC_H)$, $(AC_L-V_HC_H)-(V_LC_L-V_HC_H)$ oder $(V_LC_L-AC_H)-(V_LC_L-V_HC_H)$ ist, worin A ein menschlicher CD4-Antigen-V-Bereich ist, V_L und V_H die variablen Domänen der leichten bzw. schweren Kette eines Immunglobulins sind und C_L und

C_H die konstanten Domänen der leichten bzw. schweren Kette eines Immunglobulins sind.

24. Verfahren nach Anspruch 22 oder 23, worin das Fusionsprotein die V-J-Domäne des menschlichen CD4-Antigens umfaßt.
25. Verfahren nach Anspruch 22 oder 23, worin das menschliche CD4-Antigen die Sequenz Asn-Lys-Val-Val-Leu-Gly-Lys-Lys umfaßt.
26. Verfahren nach einem der Ansprüche 22 bis 25, worin die Immunglobulinsequenzen von IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD oder IgM stammen.
27. Verfahren nach Anspruch 26, worin die Immunglobulinsequenzen von IgG-1 oder IgG-3 stammen.
28. Verfahren nach Anspruch 26, worin die Immunglobulinsequenzen von IgA oder IgM stammen.
29. Verfahren zur Herstellung von Nukleinsäure, die für ein Polypeptid nach einem der Ansprüche 1 bis 11 kodiert.
30. Verfahren zur Herstellung eines replizierbaren Vektors, der die nach dem Verfahren nach Anspruch 29 hergestellte Nukleinsäure umfaßt.
31. Rekombinante Wirtszelle, umfassend die nach dem Verfahren nach Anspruch 29 hergestellte Nukleinsäure oder den nach dem Verfahren nach Anspruch 30 hergestellten rekombinanten Vektor.
32. Verfahren zur Herstellung eines Polypeptids nach einem der Ansprüche 1 bis 11, umfassend die Gewinnung des Polypeptids aus der Kultur einer rekombinanten Wirtszelle nach Anspruch 31.
33. Verfahren zur Herstellung von Nukleinsäure, die für ein Polypeptid nach einem der Ansprüche 12 bis 17 kodiert.
34. Verfahren zur Herstellung eines replizierbaren Vektors, der die nach dem Verfahren nach Anspruch 33 hergestellte Nukleinsäure umfaßt.
35. Rekombinante Wirtszelle, die die nach dem Verfahren nach Anspruch 33 hergestellte Nukleinsäure oder den nach dem Verfahren nach Anspruch 34 hergestellten rekombinanten Vektor umfaßt.
36. Verfahren zur Herstellung eines Polypeptids nach einem der Ansprüche 12 bis 17, umfassend die Gewinnung des Polypeptids aus der Kultur einer rekombinanten Wirtszelle nach Anspruch 35.
37. Verfahren zur Herstellung von Nukleinsäure, die für ein Polypeptid nach Anspruch 18 oder 19 kodiert.
38. Verfahren zur Herstellung einer CD4-Polypeptid-Aminosäuresequenzvariante nach Anspruch 18 oder 19, umfassend das Gewinnen der Variante aus der Kultur einer Wirtszelle, die mit der nach dem Verfahren nach Anspruch 37 hergestellten Nukleinsäure transfiziert ist.
39. Verfahren zur Herstellung von Nukleinsäure, die für eine CD4-Polypeptid-Variante nach einem der Ansprüche 22 bis 29 kodiert.
40. Verfahren zur Herstellung einer CD4-Polypeptidvariante, umfassend das Transfizieren einer Wirtszelle, die Nukleinsäure exprimiert, welche für ein Immunglobulin mit einem variablen Bereich kodiert, der gegen ein vorbestimmtes Antigen gerichtet ist, mit Nukleinsäure, die für ein Fusionsprotein kodiert, in dem ein Polypeptid, das einen variablen (V-) menschlichen CD4-Antigen-Bereich umfaßt, an seinem C-Terminus mit dem N-Terminus eines Polypeptids fusioniert ist, das einen konstanten Bereich einer Immunglobulinkette umfaßt.
41. Verfahren nach Anspruch 40, worin die CD4-Polypeptid-Variante aus der Wirtszellenkultur gewonnen wird.

Revendications

1. Procédé qui comprend la fabrication d'un polypeptide comprenant la séquence du domaine extracellulaire d'un

membre de surface cellulaire de la superfamille du gène de l'immunoglobuline, lequel membre de la superfamille n'est pas un antigène d'histocompatibilité majeur de classe I ou de classe II, une immunoglobuline, ni une chaîne α , β , γ ou δ d'un récepteur de cellules T, fusionné à une région de contact d'immunoglobuline.

5 2. Procédé selon la revendication 1 ou le polypeptide est

- (a) AC_L ;
- (b) AC_L-AC_L ;
- (c) $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ ou } V_LC_L-V_HC_H]$;
- (d) $AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ ou } V_LC_L-V_HC_H]$;
- (e) $AC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ ou } V_LC_L-V_HC_H]$;
- (f) $V_LC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, \text{ ou } V_LC_L-V_HC_H]$;
- (g) $[A-Y]_n-[V_LC_L-V_HC_H]_2$

15 où A est undit domaine extracellulaire, V_L , V_H , C_L et C_H représentent les domaines constants ou variables de chaînes lourdes ou légères d'une immunoglobuline; n est un entier; et Y désigne le résidu d'un agent de réticulation covalent.

20 3. Procédé selon la revendication 2 où les domaines V_L et V_H sont capables de se lier à un antigène prédéterminé.

4. Procédé selon l'une quelconque des revendications précédentes où la séquence d'immunoglobuline est obtenue à partir de IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD ou IgM.

25 5. Procédé selon l'une quelconque des revendications précédentes dans lequel le membre de la superfamille est CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, une molécule d'adhésion neurale ou intracellulaire, un récepteur poly-Ig, une glycoprotéine associée à une myéline, un récepteur d'IgE à affinité élevée, un récepteur d'un facteur de croissance dérivé des plaquettes, un récepteur du facteur-1 stimulant les colonies, les récepteurs gamma de Fc ou l'antigène carcinoembryonnaire.

30 6. Procédé selon la revendication 5 où le membre de la superfamille est CD2, CD4, CD8, CD28, la chaîne γ , δ ou ϵ de CD3 ou le récepteur d'IgE à affinité élevée.

7. Procédé selon la revendication 6 où le membre de la superfamille est CD4.

35 8. Procédé selon l'une quelconque des revendications précédentes dans lequel dans le polypeptide ledit domaine extracellulaire est fusionné à son terminus C au terminus N de ladite région constante.

9. Procédé selon la revendication 8 dans lequel les domaines V_1V_2 ou $V_1V_2V_3V_4$ de CD4 sont fusionnés à leurs terminus C à ladite région constante.

40 10. Procédé selon l'une quelconque des revendications précédentes où la région constante d'immunoglobuline provient d'une chaîne lourde d'immunoglobuline.

45 11. Procédé selon l'une quelconque des revendications 1 à 9 où la région constante d'immunoglobuline provient d'une chaîne légère d'immunoglobuline.

12. Procédé qui comprend la fabrication d'un polypeptide comprenant un domaine extracellulaire de CD4 fusionné à un polypeptide étranger à CD4.

50 13. Procédé selon la revendication 12 où le polypeptide étranger comprend une séquence signal.

14. Procédé selon la revendication 12 ou la revendication 13 dans lequel le polypeptide étranger est capable d'élire une réponse immune humorale chez un animal.

55 15. Procédé selon la revendication 14 dans lequel le polypeptide étranger est un polypeptide viral ou un allergène.

16. Procédé selon la revendication 12 dans lequel le polypeptide étranger est l'albumine, une apolipoprotéine ou une transferrine.

17. Procédé selon la revendication 12 dans lequel le polypeptide étranger est un polypeptide cytotoxique.
18. Procédé qui comprend la fabrication d'un variant d'une séquence d'acides aminés du polypeptide CD4 (a) ayant un domaine transmembranaire modifié pour être incapable de venir se loger dans la membrane cellulaire, ou (b) manquant d'un domaine transmembranaire.
19. Procédé selon la revendication 18 dans lequel le domaine transmembranaire a été modifié par substitution d'une séquence d'acides aminés ayant un profil d'hydropathie substantiellement hydrophile.
20. Procédé qui comprend la fabrication d'une composition comprenant un variant d'une séquence d'acides aminés du polypeptide CD4 (a) ayant un domaine transmembranaire modifié pour être incapable d'un ancrage de cellules membranaires, ou (b) manquant d'un domaine transmembranaire.
21. Procédé selon la revendication 20 dans lequel le variant comprend une séquence d'acides aminés de CD4 capable de lier gp120.
22. Procédé qui comprend la fabrication d'un variant d'un polypeptide CD4 comprenant une protéine de fusion où un polypeptide comprenant une région variable (V) d'antigène de CD4 humain est fusionné à son terminus C au terminus N d'un polypeptide comprenant une région constante d'une chaîne d'immunoglobuline associée de façon covalente à une paire chaîne lourde-chaîne légère d'immunoglobuline compagne portant un site de combinaison d'anticorps $V_L V_H$ capable de se lier à un antigène prédéterminé.
23. Procédé selon la revendication 22 dans lequel le polypeptide du variant de CD4 est $AC_H-(V_L C_L-V_H C_H)$, $(AC_L-AC_H)-(V_L C_L-V_H C_H)$, $(AC_L-V_H C_H)-(V_L C_L-V_H C_H)$, ou $(V_L C_L-AC_H)-(V_L C_L-V_H C_H)$, où A est une région V d'antigène de CD4 humain, V_L et V_H sont les domaines variables d'une chaîne légère et lourde d'immunoglobuline, respectivement, et C_L et C_H sont les domaines constants d'une chaîne légère et lourde d'immunoglobuline, respectivement.
24. Procédé selon la revendication 22 ou 23 où la protéine de fusion comprend le domaine V-J de l'antigène CD4 humain.
25. Procédé selon la revendication 22 ou la revendication 23 dans lequel l'antigène CD4 humain comprend la séquence Asn-Lys-Val-Val-Leu-Gly-Lys-Lys.
26. Procédé selon l'une quelconque des revendications 22 à 25 où les séquences d'immunoglobuline proviennent de IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD ou IgM.
27. Procédé selon la revendication 26 où les séquences d'immunoglobuline proviennent de IgG-1 ou IgG-3.
28. Procédé selon la revendication 26 dans lequel les séquences d'immunoglobuline proviennent de IgA ou IgM.
29. Procédé qui comprend la fabrication d'un acide nucléique encodant un polypeptide selon l'une quelconque des revendications 1 à 11.
30. Procédé qui comprend la fabrication d'un vecteur répliquable comprend un acide nucléique préparé par le procédé selon la revendication 29.
31. Cellule hôte recombinante comprenant un acide nucléique préparé par le procédé de la revendication 29 ou le vecteur recombinant préparé par le procédé de la revendication 30.
32. Méthode de préparation d'un polypeptide selon l'une quelconque des revendications 1 à 11 comprenant la récupération du polypeptide de la culture d'une cellule hôte recombinante selon la revendication 31.
33. Procédé qui comprend la fabrication d'un acide nucléique encodant un polypeptide selon l'une quelconque des revendications 12 à 17.
34. Procédé qui comprend la fabrication d'un vecteur répliquable comprenant un acide nucléique préparé par le procédé de la revendication 33.

35. Cellule hôte recombinante comprenant un acide nucléique préparé par le procédé de la revendication 33 ou le vecteur recombinant préparé par le procédé de la revendication 34.
- 5 36. Méthode de préparation d'un polypeptide selon l'une quelconque des revendications 12 à 17 comprenant la récupération du polypeptide de la culture d'une cellule hôte recombinante selon la revendication 35.
37. Procédé qui comprend la fabrication d'un acide nucléique encodant un polypeptide selon la revendication 18 ou la revendication 19.
- 10 38. Méthode pour préparer un variant d'une séquence d'acides aminés du polypeptide CD4 selon la revendication 18 ou la revendication 19 comprenant la récupération du variant de la culture d'une cellule hôte transfectée avec l'acide nucléique préparé par le procédé de la revendication 37.
- 15 39. Procédé qui comprend la fabrication d'un acide nucléique encodant un variant d'un polypeptide CD4 selon l'une quelconque des revendications 22 à 29.
- 20 40. Méthode de préparation d'un variant d'un polypeptide CD4 comprenant la transfection d'une cellule hôte exprimant un acide nucléique encodant une immunoglobuline ayant une région variable dirigée contre un antigène prédéterminé, avec l'acide nucléique encodant une protéine de fusion où un polypeptide comprenant une région variable (V) d'antigène de CD4 humain est fusionné à son terminus C au terminus N d'un polypeptide comprenant une région constante d'une chaîne d'immunoglobuline.
- 25 41. Méthode selon la revendication 40 dans laquelle le variant du polypeptide CD4 est récupéré de la culture de cellules hôtes.

101 AGCACCTTGGTCTGGTCAACTGGCGGTCTCTCCAGAGCCACTCAGGGAAACAAAGTGGTGGTGGGAAAAAAGGGGATACAGTGGAACTGACCTTCCTGTAACGAGAACCCAGCGTGTACCGGAGGAGGTCTGGTGGTCTTTGTTCCACGACGACCCCTTTCCCTATGTACCTGACTGGATG ArgHisLeuLeuLeuValLeuGlnLeuAlaLeuLeuProAlaAlaThrGlnGlyAsnLysValValLeuGlyLysGlyAsnThrValGlnLeuThrCys

17 ThrAlaSerGlnLysLysSerIleGlnPheHisTrpLysAsnSerAsnGlnIleLysIleLeuGlyAsnGlnGlySerPheLeuThrLysGlyProSer

301 CAAGCTGAATCATCCGCTGACTCAAGAGAGCCCTTGGGACCAAGGAACTTTCCCTGATCATCAAGAACTCTTAAGATAGAAGACTCAGATACACTTAC GTTCGACTTACTAGCGGACTGAGTCTCTTCGGAACCTGGTCTCTTGAAGGGGACTAGTAGTCTTAGAATCTCTAGTCTCTGAGTCTATGAATG LysLeuAsnAspArgAlaAspSerArgSerLeuTrpAspGlnGlyAsnPheProLeuIleLysLeuLeuLysIleGluAspSerAspThrTrp

401 ATCTGTGAAGTGGAGGACCAAGAGGAGGTGCAATGGTAGTGTTCGAGATTGACTGCCAACTCTGACACCCACTCTGCTTCAGGCGAGGAGCTTGACCC TAGACACTTCACTCTCTGCTCTCTCTCCACGTTAACCATCACAAGCTTAAGTACGCTTGAGACTGTGGTGGAGCAAGTCCCGTCTCGGACTGGG IleCysGluValGluAspGlnLysGluGluValGlnLeuLeuValPheGlyLeuThrAlaAsnSerAsnThrHisLeuLeuGlnGlyGlnSerLeuThrLeu

501 TGACCTTGGAGAGCCCTCTGCTGCTAGTAGCCCTCAGTGGCAATGTAGGAGTCCAAAGGGGTAAAAACATACAGGGGGGAGACCCCTCTCCGTGTCTCAGCT ACTGGAACCTCTCGGGGGGACCATCATCGGGGAGTCACTTACATCTCTCAGGTCTCCCAATTTTGTATGTCTCCCTCCCTCTGGGAGAGCGCATAGTCGA ThrLeuGluSerProProGlySerSerProSerValGlnCysArgSerProArgGlyLysAsnIleGlnGlyGlyLysThrLeuSerValSerGlnLeu

Fig. 10

[illegible]

Fig. 1b

1201	CTCGGGACAGGTCCTGCTGGAATCCAAACATCAAGGTTCTGCCACATGGTCCACCCGAGCTTTAAATCCGGTATGTTATCATCAGTCTAAATTCGTTAACCGCA	avai	alwNI	hinfI	saug6I	avaiI	alul	mseI	scrFI
350	SerGlyGlnValLeuLeuGluSerAsnIleLysValLeuProThrtrpSerThrProSerPheAsnAlaValTyrHisSerOC*	ppuMI	ecoO		avaiI	avaiI			
1301	GTCAGGCACCGGTGATGAATCTAACAAATCGCTCATCGCTACCTCGGCACCGTACCCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCC	nlaIV	bani	hinfI	hhaI	hinfI	hhaI	hhaI	hhaI
1401	CCCCGAGAACCGCCCTA	mnli	haeIII	saug6I	mnli	mnli	mnli	mnli	mnli

1
 2
 3
 4
 5
 6
 7
 8
 9
 10
 11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
 66
 67
 68
 69
 70
 71
 72
 73
 74
 75
 76
 77
 78
 79
 80
 81
 82
 83
 84
 85
 86
 87
 88
 89
 90
 91
 92
 93
 94
 95
 96
 97
 98
 99
 100
 101
 102
 103
 104
 105
 106
 107
 108
 109
 110
 111
 112
 113
 114
 115
 116
 117
 118
 119
 120
 121
 122
 123
 124
 125
 126
 127
 128
 129
 130
 131
 132
 133
 134
 135
 136
 137
 138
 139
 140
 141
 142
 143
 144
 145
 146
 147
 148
 149
 150
 151
 152
 153
 154
 155
 156
 157
 158
 159
 160
 161
 162
 163
 164
 165
 166
 167
 168
 169
 170
 171
 172
 173
 174
 175
 176
 177
 178
 179
 180
 181
 182
 183
 184
 185
 186
 187
 188
 189
 190
 191
 192
 193
 194
 195
 196
 197
 198
 199
 200
 201
 202
 203
 204
 205
 206
 207
 208
 209
 210
 211
 212
 213
 214
 215
 216
 217
 218
 219
 220
 221
 222
 223
 224
 225
 226
 227
 228
 229
 230
 231
 232
 233
 234
 235
 236
 237
 238
 239
 240
 241
 242
 243
 244
 245
 246
 247
 248
 249
 250
 251
 252
 253
 254
 255
 256
 257
 258
 259
 260
 261
 262
 263
 264
 265
 266
 267
 268
 269
 270
 271
 272
 273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 293
 294
 295
 296
 297
 298
 299
 300
 301
 302
 303
 304
 305
 306
 307
 308
 309
 310
 311
 312
 313
 314
 315
 316
 317
 318
 319
 320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334
 335
 336
 337
 338
 339
 340
 341
 342
 343
 344
 345
 346
 347
 348
 349
 350
 351
 352
 353
 354
 355
 356
 357
 358
 359
 360
 361
 362
 363
 364
 365
 366
 367
 368
 369
 370
 371
 372
 373
 374
 375
 376
 377
 378
 379
 380
 381
 382
 383
 384
 385
 386
 387
 388
 389
 390
 391
 392
 393
 394
 395
 396
 397
 398
 399
 400
 401
 402
 403
 404
 405
 406
 407
 408
 409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463
 464
 465
 466
 467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513
 514
 515
 516
 517
 518
 519
 520
 521
 522
 523
 524
 525
 526
 527
 528
 529
 530
 531
 532
 533
 534
 535
 536
 537
 538
 539
 540
 541
 542
 543
 544
 545
 546
 547
 548
 549
 550
 551
 552
 553
 554
 555
 556
 557
 558
 559
 560
 561
 562
 563
 564
 565
 566
 567
 568
 569
 570
 571
 572
 573
 574
 575
 576
 577
 578
 579
 580
 581
 582
 583
 584
 585
 586
 587
 588
 589
 590
 591
 592
 593
 594
 595
 596
 597
 598
 599
 600
 601
 602
 603
 604
 605
 606
 607
 608
 609
 610
 611
 612
 613
 614
 615
 616
 617
 618
 619
 620
 621
 622
 623
 624
 625
 626
 627
 628
 629
 630
 631
 632
 633
 634
 635
 636
 637
 638
 639
 640
 641
 642
 643
 644
 645
 646
 647
 648
 649
 650
 651
 652
 653
 654
 655
 656
 657
 658
 659
 660
 661
 662
 663
 664
 665
 666
 667
 668
 669
 670
 671
 672
 673
 674
 675
 676
 677
 678
 679
 680
 681
 682
 683
 684
 685
 686
 687
 688
 689
 690
 691
 692
 693
 694
 695
 696
 697
 698
 699
 700
 701
 702
 703
 704
 705
 706
 707
 708
 709
 710
 711
 712
 713
 714
 715
 716
 717
 718
 719
 720
 721
 722
 723
 724
 725
 726
 727
 728
 729
 730
 731
 732
 733
 734
 735
 736
 737
 738
 739
 740
 741
 742
 743
 744
 745
 746
 747
 748
 749
 750
 751
 752
 753
 754
 755
 756
 757
 758
 759
 760
 761
 762
 763
 764
 765
 766
 767
 768
 769
 770
 771
 772
 773
 774
 775
 776
 777
 778
 779
 780
 781
 782
 783
 784
 785
 786
 787
 788
 789
 790
 791
 792
 793
 794
 795
 796
 797
 798
 799
 800
 801
 802
 803
 804
 805
 806
 807
 808
 809
 810
 811
 812
 813
 814
 815
 816
 817
 818
 819
 820
 821
 822
 823
 824
 825
 826
 827
 828
 829
 830
 831
 832
 833
 834
 835
 836
 837
 838
 839
 840
 841
 842
 843
 844
 845
 846
 847
 848
 849
 850
 851
 852
 853
 854
 855
 856
 857
 858
 859
 860
 861
 862
 863
 864
 865
 866
 867
 868
 869
 870
 871
 872
 873
 874
 875
 876
 877
 878
 879
 880
 881
 882
 883
 884
 885
 886
 887
 888
 889
 890
 891
 892
 893
 894
 895
 896
 897
 898
 899
 900
 901
 902
 903
 904
 905
 906
 907
 908
 909
 910
 911
 912
 913
 914
 915
 916
 917
 918
 919
 920
 921
 922
 923
 924
 925
 926
 927
 928
 929
 930
 931
 932
 933
 934
 935
 936
 937
 938
 939
 940
 941
 942
 943
 944
 945
 946
 947
 948
 949
 950
 951
 952
 953
 954
 955
 956
 957
 958
 959
 960
 961
 962
 963
 964
 965
 966
 967
 968
 969
 970
 971
 972
 973
 974
 975
 976
 977
 978
 979
 980
 981
 982
 983
 984
 985
 986
 987
 988
 989
 990
 991
 992
 993
 994
 995
 996
 997
 998
 999
 1000

Fig. 2a

[illegible]

Fig. 26

```

                                mnli
                                ddei
                                mstII
                                eco8II
                                foki
                                alwNI
                                ddei
                                hinfi
                                plei
                                avai
                                alwNI
1201 CTGAGAACAGGAGCGCAAGCTCTCGAAGCGGAGAGCGGTGGGTGCTGAACCTGAGCGGGGATGTGGCAGTGTCTGCTAGTACTCGGGAC
    GACCTCTTGTCTCCTCCGTTTCCAGAGCTCGCCCTCTTCCAGAGCTCGCCCTCTTCCAGAGCTCGCCCTCTTCCAGAGCTCGCCCTCTTCCAGAGCTCGCCCTG
376 LeuGluasnLysGluAlaLysValSerLysArgGluLysAlaValTrpValLeuAsnProGluAlaGlyMetTrpGlnCysLeuLeuSerAspSerGlyGln
                                sau96I
                                avaiI
                                ppvMI
                                hinfI
                                nlaIII
                                aluI
                                msel
                                nlaIV
                                banI
1301 AGTCTGTCTGGAATCCAAACATCAAGCTTCTGCCACATGTGTCCACCCCGAGCTTTAATGCGGTAGTTTATCAGTATAAATTGCTAACGCCAGTCAGGCA
    TCCAGACGACCTTAGCTTGTAGTTCAGAGCGGTGTACAGAGGTGGGCTCGAAATACGCCCATCAAAATAGTGTCAATTTAACGATTCCGTCAGTCCGT
410 ValLeuLeuGluSerAsnIleLysValLeuProThrTrpSerThrProSerPheAsnAlaValValtyHisSerOC*
                                haeIII
                                sau96I
                                scrFI
                                rsai
                                nciI
                                mspi
                                hpaII
                                hpaII
1401 CCGTGTATGAATCTAACAATGGCTCATCTCCTCGGACCGGTACCCCTGGTGTAGGCATAGCTTGGTTATGCCGTACTGCCGGGCTCT
    GCCACATACCTTAGATTCTTACCGCGAGTAGCAGTAGGAGCGGTGGCAGTGGGACCTACGACATCCGTATCCGAACCAATACGGCCCATGACGGCCCGGAGA
1501 TCGGGGAT
    ACGCCCTA

```

Fig. 2c

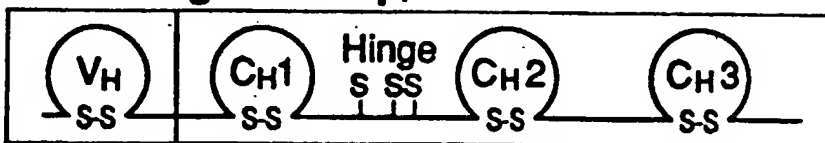
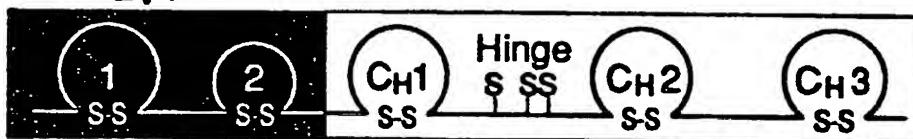
CD4**Immunoglobulin γ_1** **Soluble rCD4****CD4₂ γ_1** **CD4₄ γ_1** 

Fig.3

1 GAATTCGTCACTGCGCGGACACGCGCGGTATATTACTGTGCGAGAGCCACCTTTTGCCTATGTACAGGAGGCTCCCTTGTGGATCGACCCCTTGG
 CTTAAGACAGTACGCGCGCTGTGCGGCATATATACACAGCTTCGGTGGAAACGGATACCATGTCTCCCTCGAGGGGGAACAACCTAGCTGGGGACC
 ValThrAlaAlaAspThrAlaValTyrCysAlaAlaGAlaThrPheCysLeuTrpYrArgGluArgProProCysTrpIleAspProTirp
 72
 101 GGCCTGGGAACCCCTGGTCAACCGTCTCTCGGCGCTCCACCAAGGGCCCATCGGTCTTCCCTCGACCTCTCCCAAGACACCTCTGGGGGACACAGCGG
 CCGACCCCTGGGACCAAGTGGCAGAGCGGAGGTTCCTCGGTAGCCAGAGGGGACCTGGTCTCGTGGAGACCCCTCGTCTCGCC
 103 GlyLeuGlyThrLeuValThrValSerSerAlaSerThrLysGlyProSerValPheProLeuAlaProSerSerLysSerThrSerGlyGlyThrAlaAla
 11
 120 ddeI mstII pIeI fnu4HI hphI mspI tthIII hpaII hgiAI mspI hgiAI bspI286 hpaII hgiAI mspI hgiAI
 137 LeuGlyCysLeuValLysAspTyrPheProGluProValThrValSerSerLysSerThrPheProAlaValLeu
 140
 150 ddeI mstII pIeI fnu4HI hphI mspI tthIII hpaII hgiAI mspI hgiAI bspI286 hpaII hgiAI mspI hgiAI
 160
 170 GlnSerSerGlyLeuTyrSerLeuSerSerValValThrValProSerSerSerLeuGlyThrGlnThrTyrIleCysAsnValAsnHisLysProSer
 180
 190
 201 CCCTGGGCTGCCTGGTCAAGGACTACTTCCCGAACGGGTGACGGTGTCTGTGGACTCAGCGCCCTGACCAAGCGGCTGACACCTTCCCGGCTGTCT
 GGGACCCGAGCAGCAGTCTGATGAAGGGCTTGGCCACTGCACAGCACCTGAGTCCGGGACTGTCGGGACCTGTCGGGAGGCGGACAGGA
 203 AsnThrLysValAspLysValGluProLysSerCysAspLysThrHisThrCysProProCysProAlaProGluLeuLeuGlyGlyProSerValPhe
 210
 220
 230
 240
 250
 260
 270
 280
 290
 300
 310
 320
 330
 340
 350
 360
 370
 380
 390
 400
 410
 420
 430
 440
 450
 460
 470
 480
 490
 500
 510
 520
 530
 540
 550
 560
 570
 580
 590
 600
 610
 620
 630
 640
 650
 660
 670
 680
 690
 700
 710
 720
 730
 740
 750
 760
 770
 780
 790
 800
 810
 820
 830
 840
 850
 860
 870
 880
 890
 900
 910
 920
 930
 940
 950
 960
 970
 980
 990
 1000

Fig. 4a

Fc

[illegible]

Fig. 4b

sau96I
 avaiI
 nlaIV
 mnlI styI
 fnu4HI
 pstI
 haeIII
 fnu4HI bbvI mboII
 hphI
 1 GAATTCACCTCACCATCAGCGGCTGCAGCCTGAAGATTTTGCACACTTATTACTGCCAACAGTATAAGAGTTTGTGCTCACTTTCCGGCGGAGGACCA
 CTTAAGTGAGAGTGGTAGTCGCCGCGGACGTCGGACTTCTAAACGTTGAATAATGAGGTTGTATATTCTCAACACAGCGAGTGAAAGCCCTCCCTGGT
 72 ThrLeuThrIleSerGlyLeuGlnProGluAspPheAlaThrTyrCysGlnGlnTyrLysSerLeuSerLeuThrPheGlyGlyGlyThrLys
 sau3AI
 dpnI
 fnu4HI
 bbvI
 mboII mboII
 101 AGGTGGAGATCAAAACGAACGTGTGGCTGCACCATCTGTCTTCATCTTCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCCTCTGTTGTGCTGCTGT
 TCCACCTCTAGTTGCTTGACACCGACGTCGGTAGACAGAGTAGAAGGCGGTAGACTACTCGTCAACTTTAGACCTTGACGGAGACAACACACGGACCGA
 104 ValGluIleLysArgThrValAlaAlaProSerValPheIlePheProSerAspGluGlnLeuLysSerGlyThrAlaSerValValCysLeuLeu
 ← $V_K T_K$ C_K →
 haeI
 haeIII
 xmnI
 mnlI
 rsaI
 mnlI
 201 GAATAACTTCTATCCCGAGAGAGCCCAAGTACAGTGAAGTGGATAACGCCCTCCCAATCGGGTAACTCCCGAGGAGAGTGTACAGAGCAGGACAGCAAG
 CTTATTGAAGATAGGGTCTCTCCGGTTTCATGTACCTCCACCTATTGGGGAGGTTAGCCCATTTGAGGGTCTCTCACAGTGTCTCGTCTGTGCTTC
 137 AsnAsnPheTyrProArgGluAlaLysValGlnTrpLysValAspAsnAlaLeuGlnSerGlyAsnSerGlnGluSerValThrGluGlnAspSerLys
 fnu4HI
 ddeI
 mnlI bbvI
 hgaI
 301 GACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAGCAGACTACGAGAAACACAAAGTCTACGCCCTGCCAAGTCAACCCATCAGGGCTGAGCT
 CTGTCGTGGATGTCGGAGTCGTCGGGACTGCGACTCGTTTCGCTGATGCTCTTTGTGTTTCAGATCGGGACGCTTCAGTGGGTAGTCCCGGACTCGA
 170 AspSerThrTyrSerLeuSerSerThrLeuThrLeuSerLysAlaAspTyrGluLysHisLysValTyrAlaCysGluValThrHisGlnGlyLeuSerSer
 aluI
 mnlI
 bsp1286 bspMI mnlI
 401 CGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTAGAGGGAGAGTGTCCCCCACCTGTCTCCTCAGT
 GCGGGCAGTGTCTCGAAGTGTCCCTCTCACAAATCTCCCTCTTCACGGGGGTGGACGAGGAGTCA
 204 ProValThrLysSerPheAsnArgGlyGluCysAM*
 haeIII
 sau96I aluI
 ecoO banII
 hphI
 alwNI ddeI
 accI
 haeIII
 hgiAI
 bsp1286
 ddeI

Fig. 5

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 314 317 B2

(12)

NEW EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the opposition decision:
23.03.2005 Bulletin 2005/12

(51) Int Cl.7: **C12N 15/00**, C12P 21/02,
A61K 38/10
// G01N33/566

(45) Mention of the grant of the patent:
26.08.1998 Bulletin 1998/35

(21) Application number: **88309194.4**

(22) Date of filing: **03.10.1988**

(54) Adheson variants, nucleic acid encoding them and compositions comprising them

Adhäsionsvarianten, dafür kodierende Nukleinsäure und diese enthaltende Zusammensetzungen
Variantes d'adhésions, acide nucléique les codant ainsi que compositions les contenant

(84) Designated Contracting States:
ES

(30) Priority: 02.10.1987 US 104329
28.09.1988 US 250785

(43) Date of publication of application:
03.05.1989 Bulletin 1989/18

(73) Proprietor: Genentech Inc.
South San Francisco, CA 94080-4990 (US)

(72) Inventors:
• Capon, Daniel J.
San Mateo California 94402 (US)
• Gregory, Timothy J.
Hillsborough California 94010 (US)

(74) Representative: Armitage, Ian Michael et al
Mewburn Ellis LLP
York House
23 Kingsway
London WC2B 6HP (GB)

(56) References cited:
WO-A-88/01304

- MADDON P J ET AL: "THE ISOLATION AND NUCLEOTIDE SEQUENCE OF A CDNA ENCODING THE T CELL SURFACE PROTEIN T4: A NEW MEMBER OF THE IMMUNOGLOBULIN GENE FAMILY" CELL, CELL PRESS, CAMBRIDGE, NA, US, vol. 42, no. 1, 1 August 1985 (1985-08-01), pages 93-104, XP002051712 ISSN: 0092-8674
- CLARK S J ET AL: "PEPTIDE AND NUCLEOTIDE SEQUENCES OF RAT CD4 (W3/25) ANTIGEN: EVIDENCE FOR DERIVATION FROM A STRUCTURE WITH FOUR IMMUNOGLOBULIN-RELATED DOMAINS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 84, 1 March 1987 (1987-03-01), pages 1649-1653, XP002051713 ISSN: 0027-8424
- D.H.SMITH ET AL: "Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen" SCIENCE, vol. 238, 18 December 1987 (1987-12-18), pages 1704-1707, XP002964490

EP 0 314 317 B2

Description

[0001] This application relates to compositions for antiviral or immunomodulatory therapy. In particular, it relates to compositions useful in the treatment of Human Immunodeficiency Virus (HIV) infections.

[0002] The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (H. Lane *et al.*, *Ann. Rev. Immunol.* **3**:477 [1985]). CD4 is a non-polymorphic glycoprotein with homology to the immunoglobulin gene superfamily (P. Maddon *et al.*, *Cell* **42**:93 [1985]). Together with the CD8 surface antigen, CD4 defines two distinct subsets of mature peripheral T cells (E. Reinherz *et al.*, *Cell* **19**:821 [1980]), which are distinguished by their ability to interact with nominal antigen targets in the context of class I and class II major histocompatibility complex (MHC) antigens, respectively (S. Swain, *Proc. Natl. Acad. Sci.* **78**:7101 [1981]; E. Engleman *et al.*, *J. Immunol.* **127**:2124 [1981]; H. Spitz *et al.*, *J. Immunol.* **129**:1563 [1982]; W. Biddison *et al.*, *J. Exp. Med.* **156**:1065 [1982]; and D. Wilde *et al.*, *J. Immunol.* **131**:2178 [1983]). For the most part, CD4 T cells display the helper/inducer T cell phenotype (E. Reinherz, *supra*), although CD4 T cells characterized as cytotoxic/suppressor T cells have also been identified (Y Thomas *et al.*, *J. Exp. Med.* **154**:459 [1981]; J. S. Meuer *et al.*, *Proc. Natl. Acad. Sci. USA* **79**:4395 [1982]; and A. Krensky *et al.*, *Proc. Natl. Acad. Sci. USA* **79**:2365 [1982]). The loss of CD4 helper/inducer T cell function probably underlies the profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of the acquired immunodeficiency syndrome (AIDS) (H. Lane *supra*).

[0003] Studies of HIV-1 infection of fractionated CD4 and CD8 T cells from normal donors and AIDS patients have revealed that depletion of CD4 T cells results from the ability of HIV-I to selectively infect, replicate in, and ultimately destroy this T lymphocyte subset (D. Klatzmann *et al.*, *Science* **225**:59 [1984]). The possibility that CD4 itself is an essential component of the cellular receptor for HIV-I was first indicated by the observation that monoclonal antibodies directed against CD4 block HIV-I infection and syncytia induction (A. Dalglish *et al.*, *Nature [London]* **312**:767 [1984]; J. Mc-Dougal *et al.*, *J. Immunol.* **135**:3151 [1985]). This hypothesis has been confirmed by the demonstration that a molecular complex forms between CD4 and gp120, the major envelope glycoprotein of HIV-I (J. McDougal *et al.*, *Science* **231**: 382 [1986]; and the finding that HIV-I tropism can be conferred upon ordinarily non-permissive human cells following the stable expression of a CD4 cDNA (P. Maddon *et al.*, *Cell* **47**:333 [1986]). Furthermore, the neurotropic properties of HIV-I, reflected by a high incidence of central nervous system dysfunction in HIV-I infected individuals (W. Snider *et al.*, *Ann. Neurol.* **14**:403 [1983]), and the ability to detect HIV-I in the brain tissue and cerebrospinal fluid of AIDS patients (G. Shaw *et al.*, *Science* **227**:177 [1985]; L. Epstein, *AIDS Res.* **1**:447 [1985]; S. Koenig, *Science* **233**: 1089 [1986]; D. Ho *et al.*, *N. Engl. J. Med.* **313**:1498 [1985]; J. Levy *et al.*, *Lancet* **II**:586 [1985]), appears to have its explanation in the expression of CD4 in cells of neuronal, glial and monocyte/macrophage origin (P. Maddon, *Cell* **47**: 444 [1986]; I. Funke *et al.*, *J. Exp. Med.* **165**:1230 [1986]; B. Tourville *et al.*, *Science* **234**:610 [1986]).

[0004] In addition to determining the susceptibility to HIV-I infection, the manifestation of cytopathic effects in the infected host cell appears to involve CD4. Antibody to CD4 was found to inhibit the fusion of uninfected CD4 T cells with HIV-I infected cells *in vitro*; moreover, the giant multinucleated cells produced by this event die shortly after being formed resulting in the depletion of the population of CD4 cells (J. Lifson *et al.*, *Science* **232**:1123 [1986]). Formation of syncytia also requires gp120 expression, and can be elicited by coculturing CD4-positive cell lines with cell lines expressing the HIV-I *env* gene in the absence of other viral structural or regulatory proteins (J. Sodroski *et al.*, *Nature* **322**:470 [1986]; J. Lifson *et al.*, *Nature* **323**:725 [1986]). Thus, in mediating both the initial infection by HIV-I as well as eventual cell death, the interaction between gp120 and CD4 constitutes one of several critical entry points in the viral life cycle amenable to therapeutic intervention (H. Mitsuya *et al.*, *Nature* **325**:773 [1987]).

[0005] The known sequence of the CD4 precursor predicts a hydrophobic signal peptide, an extracellular region of approximately 370 amino acids, a highly hydrophobic stretch with significant identity to the membrane-spanning domain of the class II MHC beta chain, and a highly charged intracellular sequence of 40 residues (P. Madden, *Cell* **42**:93 [1985]). The extracellular domain of CD4 consists of four contiguous regions each having amino acid and structural similarity to the variable and joining (V-J) domains of immunoglobulin light chains as well as related regions in other members of the immunoglobulin gene superfamily (a subclass of which are defined herein by the coined term "adhesons". These structurally similar regions of CD4 are termed the V₁J₁, V₂J₂, V₃J₃ and V₄J₄ domains (denominated 1-4 in Fig. 3).

[0006] A successful strategy in the development of drugs for the treatment of many receptor mediated abnormalities has been the identification of antagonists which block binding of the natural ligand. Since the CD4 adheson ordinarily binds to the recognition sites of the HIV envelope it would appear to be a candidate for therapeutically sequestering these HIV sites, thereby blocking viral infectivity. However, full length CD4 and other adhesons are cell membrane proteins which are anchored in the lipid bilayer of lymphocytes. The presence of membrane components will be undesirable from the standpoint of manufacturing and purification. In addition, since adhesons are normally present only on cell surfaces, it would be desirable to produce adhesons in a form which is more stable in the circulation. Additionally, even truncated, soluble CD4 adheson (generally referred to as CD4T) may not be optimally effective as a therapeutic

since it possesses a relatively short biological half-life, binds to HIV no better than cell surface CD4, may not cross the placental or other biological barriers and since it merely sequesters the HIV recognition sites without in itself bearing an infected-cell killing or virus killing functionality.

[0007] Accordingly, it is desirable to produce soluble, secreted adhesions. It is also desirable to produce CD4 derivatives useful in the treatment of AIDS and related conditions, in a manner essentially unaffected by the extreme degree of genetic variation observed among various HIV-1 isolates and their respective *env* polypeptides (J. Coffin, Cell 46:1 [1986]). It is further desirable to prepare adhesions fused to other polypeptides in order to provide molecules with novel functionalities such as those described above for therapeutic use, or diagnostic reagents for the *in vitro* assay of adhesions or their ligands. In particular, it is desirable to prepare molecules for directing toxins or effector molecules (for example the Fc domain of immunoglobulin) to cells bearing receptors for the adhesions, e.g. HIV gp120 in the case of CD4, and for use in facilitating purification of the adhesions. It is further desirable to provide stable, highly purified adhesion preparations.

[0008] The present invention provides nucleic acid encoding an amino acid sequence variant of an adhesion (CD4), in particular a variant in which the trans-membrane domain is modified so that it is no longer capable of becoming lodged in the cell membrane, i.e. inactivated, for example, by deletion or substitution.

[0009] Variant adhesions are produced by a method comprising (a) transforming a host cell with nucleic acid encoding an amino acid sequence variant of an adhesion, (b) culturing the host cell and (c) recovering the variant adhesion from the host cell culture media.

[0010] In another embodiment the invention provides an adhesion variant selected from the group consisting of (a) a CD4 adhesion amino acid sequence variant having an inactivated transmembrane domain and (b) a polypeptide comprising an adhesion extracellular domain fused to the sequence of a polypeptide which is extraneous to the adhesion, this latter for CD4 extracellular domain being for example selected from a cytotoxin, an immunogen or an immunoglobulin constant domain, and for other adhesion extracellular domains being an immunoglobulin constant region.

[0011] In a preferred embodiment a polypeptide comprising a gp120 binding domain of the CD4 adhesion is fused at its C-terminus to an immunoglobulin constant domain, or is linked to a cytotoxic polypeptide such as ricin.

[0012] The CD4 adhesion variants provided herein are purified and formulated in pharmacologically acceptable vehicles for administration to patients in need of antiviral or immunomodulatory therapy, in particular patients infected with HIV.

Brief Description of the Drawings

[0013]

Figs. 1a-1c depict the amino acid and nucleotide sequence of a secreted form of the CD4 adhesion (CD4T). Other forms of CD4T terminate at Ser 366 or Pro 368. The signal processing site is designated with an arrow.

Figs. 2a-2c depict the amino acid and nucleotide sequence of a fusion of the herpes gD leader and N-terminal 27 residues to the putative mature N-terminus of CD4T.

Fig. 3 depicts the structural elements of the native and soluble CD4 antigen, the native immunoglobulin G heavy (γ) chain and two exemplary heavy chain-CD4 chimeras.

Figs. 4a-4b are a map of the linked immunoglobulin γ chain fragment employed in the preparation of CD4 fusions. Insert sites are designated $\gamma 1$ and Fc.

Fig. 5 is a map of a human light chain fragment useful for CD4 fusions at the arrow flanked by $V_k J_k$ (light variable and joining) and C_k (light constant).

Detailed Description

[0014] Adhesions are cell surface polypeptides having an extracellular domain which is of a member of the immunoglobulin gene superfamily, excluding, however, highly polymorphic members of this superfamily selected from the group of class I and class II major histocompatibility antigens, immunoglobulins and T-cell receptor α , β , γ and δ chains. Examples of adhesions include CD2, CD4, CD8, CD28, the γ , δ and ϵ chains of CD3, OX-2, Thy-1, the intercellular or neural cell adhesion molecules (I-CAM or N-CAM), neurocytoplasmic protein (NCP-3), poly-Ig receptor, myelin-associated glycoprotein (MAG), high affinity IgE receptor, the major glycoprotein of peripheral myelin (Po), platelet derived growth factor receptor, colony stimulating factor-1 receptor, macrophage Fc receptor, Fc gamma receptors and carcinoembryonic antigen. Preferred adhesions are CD4, CD8 and high affinity IgE receptor.

[0015] The present invention in one aspect provides a process which comprises making an immunoglobulin heavy chain dimer in which the extracellular domain sequence of a cell surface member of the immunoglobulin gene superfamily, which superfamily member is not a class I or class II major histocompatibility antigen, an immunoglobulin, nor a T-cell receptor α , β , γ or δ chain, is substituted for the variable region of an immunoglobulin heavy chain, which dimer

comprises a polypeptide comprising said extracellular domain fused to an immunoglobulin heavy chain constant region, and in which dimer said extracellular domain binds a ligand of said member of the immunoglobulin gene superfamily. Such an immunoglobulin heavy chain dimer may be divalent for said ligand.

[0016] In another aspect, the present invention provides a process which comprises making a polypeptide comprising an extracellular domain of CD4 fused to a polypeptide extraneous to CD4.

[0017] In another aspect, the present invention provides a process which comprises making a CD4 polypeptide amino acid sequence variant (a) having a transmembrane domain modified to be incapable of becoming lodged in the cell membrane, or (b) lacking a transmembrane domain.

[0018] Described herein are amino acid sequence variants of adhesions. Amino acid sequence variants of adhesions are prepared with various objectives in mind, including increasing the affinity of the adhesion for its binding partner, facilitating the stability, purification and preparation of the adhesion, increasing its plasma half life, improving therapeutic efficacy as described above in the background, introducing additional functionalities and lessening the severity or occurrence of side effects during therapeutic use of the adhesion. Amino acid sequence variants of adhesions fall into one or a combination of the following classes: insertional, substitutional or deletional variants.

[0019] Insertional amino acid sequence variants are those in which one or more amino acid residues extraneous to the adhesion are introduced into a predetermined site in the adhesion including the C or N termini. Such variants are referred to as fusions of the adhesion and a different polypeptide are produced. Such other polypeptides contain sequences other than those which are normally found in the adhesion at the inserted position. Several groups of fusions are described. Immunologically active adhesion fusions comprise an adhesion and a polypeptide containing a non-adhesion epitope. The non-adhesion epitope is any immunologically competent polypeptide, i.e., any polypeptide which is capable of eliciting an immune response in the animal to which the fusion is to be administered or which is capable of being bound by an antibody raised against the non-adhesion polypeptide. Typical non-adhesion epitopes will be those which are borne by allergens, autoimmune epitopes, or other potent immunogens or antigens recognized by pre-existing antibodies in the fusion recipient, including bacterial polypeptides such as trpLE, beta-galactosidase, viral polypeptides such as herpes gD protein, and the like. Immunogenic fusions are produced by cross-linking *in vitro* or by recombinant cell culture transformed with DNA encoding an immunogenic polypeptide. It is preferable that the immunogenic fusion be one in which the immunogenic sequence is joined to or inserted into the adhesion antigen or fragment thereof by a peptide bond(s). These products therefore consist of a linear polypeptide chain containing adhesion epitopes and at least one epitope foreign to the adhesion. The epitopes can be introduced anywhere within the adhesion molecule or fragment thereof. Such fusions are conveniently made in recombinant host cells or by the use of bifunctional cross-linking agents. The use of a cross-linking agent to fuse the adhesion to the immunogenic polypeptide is not as desirable as a linear fusion because the crosslinked products are not as easily synthesized in structurally homogeneous form.

[0020] These immunogenic insertions are particularly useful when formulated into a pharmacologically acceptable carrier and administered to a subject in order to raise antibodies against the adhesion, which antibodies in turn are useful in diagnostics or in purification of adhesion by immunoaffinity techniques known *per se*. Alternatively, in the purification of adhesions, binding partners for the fused non-adhesion polypeptide, e.g. antibodies, receptors or ligands, are used to adsorb the fusion from impure admixtures, after which the fusion is eluted and, if desired, the adhesion is recovered from the fusion, e.g. by enzymatic cleavage.

[0021] Other fusions, which may or may not also be immunologically active, include fusions of the adhesion sequence with a signal sequence heterologous to the adhesion, fusions of transmembrane-modified CD4 adhesions, for example, to polypeptides having enhanced plasma half life (ordinarily >about 20 hours) such as immunoglobulin chains or fragments thereof, and fusions with cytotoxic functionalities. Signal sequence fusions are employed in order to more expeditiously direct the secretion of the adhesion. The heterologous signal replaces the native adhesion signal, and when the resulting fusion is recognized, i.e. processed and cleaved by the host cell, the adhesion is secreted. Signals are selected based on the intended host cell, and may include bacterial yeast, mammalian and viral sequences. The herpes gD glycoprotein signal is suitable for use in mammalian expression systems.

[0022] Plasma proteins which have enhanced plasma half-life longer than that of transmembrane modified CD4 include serum albumin, immunoglobulins, apolipoproteins, and transferrin. Preferably, the adhesion-plasma protein fusion is not significantly immunogenic in the animal in which it is used and the plasma protein does not cause undesirable side effects in patients by virtue of its normal biological activity.

[0023] In a specific embodiment the adhesion extracellular domain is conjugated with an immunoglobulin constant region sequence. The resulting products are referred to herein as immunoadhesions. Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, *J. Immun.* 123: 793 (1979); Köhler *et al.*, *P.N.A.S. USA* 77:2197 (1980); Raso *et al.*, *Cancer Res.* 41:2073 (1981); Morrison *et al.*, *Ann. Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison *et al.*, *P.N.A.S. USA* 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S.

patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

[0024] Ordinarily, the domains of adhesions that are homologous to immunoglobulins and extracellular in their native environment are fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s)-like thereof, retaining at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Immunoglobulins and other polypeptides having enhanced plasma half life are fused to the extracellular or ligand binding domains of other adhesions in the same fashion.

[0025] The boundary domains for the CD4 V-like regions (V1-V4) are, respectively, about 100-109, about 175-184, about 289-298, and about 360-369 (based on the precursor CD4 amino acid sequence in which the initiating met is -25; Fig. 1a), CD4 sequences containing any of the CD4 V domains are fused to the immunoglobulin sequence. It is preferable that the V1 domain of CD4 or CD4 lacking the transmembrane and cytoplasmic domains be fused at their C-termini to the immunoglobulin constant region. The precise site at which the fusion is made is not critical; the boundary domains noted herein are for guidance only and other sites neighboring or within the V regions may be selected in order to optimize the secretion or binding characteristics of the CD4. The optimal site will be determined by routine experimentation. In general, it has been found that the fusions are expressed intracellularly, but a great deal of variation is encountered in the degree of secretion of the fusions from recombinant hosts. For instance, the following table demonstrates the various immunoglobulin fusions that have been obtained by the method of this invention. In all examples of CD4 immunoadhesions, the CD4 signal was used to direct secretion from 293 cells. Lower case m represents murine origin, while the lower case h designates human origin. V and C are abbreviations for immunoglobulin variable and constant domains respectively. The numerical subscripts indicate the number of parenthetical units found in the designated multimer. It will be understood that the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins. The CD4 immunoadhesions typically contained either the first N-terminal 366 residues of CD4 (CD4₄) or the first 180 N-terminal residues of CD4 (CD4₂) linked at their C-terminus to the κ (light) chain or IgG1 heavy chain constant region (γ 1).

Table I

Transfected Gene	Secreted Product
mV _{κ} C _{κ}	mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
mV _{γ1} C _{γ1}	ND
mV _{κ} C _{κ} + μ γ 1C _{γ1}	(mV _{κ} C _{κ}) ₂ (mV _{γ1} C _{γ1}) ₂ + mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
hCD4-mC _{κ}	hCD4-mC _{κ} and/or (hCD4-mC _{κ}) ₂
hCD4-mC _{γ1}	ND
hCD4-mC _{κ} + hCD4-mC _{γ1}	(hCD4-mC _{κ}) ₂ (hCD4-mC _{γ1}) ₂ + hCD4-mC _{κ} and/or (hCD4-mC _{κ}) ₂
hCD4-hC _{κ}	hCD4-hC _{κ} and/or (hCD4-hC _{κ}) ₂
hCD4-hC _{γ1}	(hCD4-hC _{γ1}) ₂
hCD4-hC _{κ} + hCD4-hC _{γ1}	(hCD4-hC _{κ}) ₂ (hCD4-hC _{γ1}) ₂ + hCD4-hC _{κ} and/or (hCD4-hC _{κ}) ₂
mV _{κ} C _{κ} + hCD4-hC _{γ1}	(mV _{κ} C _{κ}) ₂ (hCD4-hC _{γ1}) ₂ + mV _{κ} C _{κ} and/or (mV _{κ} C _{κ}) ₂
*ND - Not detected	

It is interesting to observe from this table that the CD4-human heavy chain immunoadheson was secreted as a dimer whereas the analogous murine construction was not detected (this not excluding the intracellular accumulation of the protein, however). The ability of the hCD4-hC γ 1 transformants to produce heavy chain dimer was unexpected since previous work had suggested that immunoglobulin heavy chains are not secreted unless the hosts are cotransformed with nucleic acid encoding both heavy and light chain (Valle et al., *Nature* 241:338 [1981]). According to this invention, CD4-IgG immunoadheson chimeras are readily secreted wherein the CD4 epitope is present in light chain dimers, heavy chain monomers or dimers, and heavy and light chain tetramers wherein the CD4 epitope is present fused to

one or more light or heavy chains, including heterotetramers wherein up to and including all four variable region analogues are derived from CD4. Where light-heavy chain non-CD4 variable domain is present, a heterofunctional antibody thus is provided.

[0026] Suitable companion immunoglobulin combining sites and fusion partners are obtained from IgG-1, -2, -3, or 4-subtypes, IgA, IgE, IgD or IgM, but preferably IgG-1.

[0027] A preferred embodiment is a fusion of an N-terminal portion of CD4, which contains the binding site for the gp120 envelope protein of HIV, to the C-terminal F_c portion of an antibody, containing the effector functions of immunoglobulin G₁. There are two preferred embodiments of this sort; in one, the entire heavy chain constant region is fused to a portion of CD4; in another, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG F_c chemically (residue 216, taking the first residue of heavy chain constant region to be 114 [Kabat *et al.*, "Sequences of Proteins of Immunological Interest" 4th Ed., 1987], or analogous sites of other immunoglobulins) is fused to a portion of CD4. These embodiments are described in the examples.

[0028] More particularly, those variants in which one or more immunoglobulin-like domains of an adhesion are substituted for the variable region of an immunoglobulin chain are believed to exhibit improved *in vivo* plasma half life. These chimeras are constructed in a fashion similar to chimeric antibodies in which a variable domain from a antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; Munro, Nature 312: (13 December 1984); Neuberger *et al.*, Nature 312: (13 December 1984); Sharon *et al.*, Nature 309: (24 May 1984); Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Morrison *et al.* Science 229:1202-1207 (1985); and Boulianne *et al.*, Nature 312:643-646 (13 December 1984). The DNA encoding a CD4 V-like region is cleaved by a restriction enzyme at or proximal to a DNA encoding a boundary domain and at a point at or near the DNA encoding N-terminal 5' end of the mature antigen (where use of a non-CD4 leader is contemplated) or at or proximal to the 5' end of the N-terminal coding region for the antigen (where a native CD4 signal is employed). This DNA fragment then is readily inserted into DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, tailored by deletional mutagenesis. Preferably, this is a human immunoglobulin when the variant is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams *et al.*, Biochemistry 19:2711-2719 (1980); Gough *et al.*, Biochemistry 19:2702-2710 (1980); Dolby *et al.*, P.N.A.S. USA, 77:6027-6031 (1980); Rice *et al.*, P.N.A.S. USA 79: 7862-7865 (1982); Falkner *et al.*, Nature 298:286-288 (1982); and Morrison *et al.*, Ann. Rev. Immunol. 2:239-256 (1984).

[0029] DNA encoding the immunoglobulin or immunoadheson chimeric chain(s) is transfected into a host cell for expression. If the host cell is producing a heavy chain immunoglobulin prior to transfection then one need only transfect with the adhesion fused light chain to produce a heteroantibody. Similarly, if the host cell is expressing a light chain then DNA encoding a heavy chain adhesion fusion is transfected to produce a heteroantibody. The aforementioned immunoglobulins having one or more arms bearing the adhesion domain and one or more arms bearing companion variable regions result in dual specificity for adhesion ligand and for an antigen. These are produced by the above-described recombinant methods or by *in vitro* procedures. In the latter case, for example, F(ab')₂ fragments of the adhesion fusion and an immunoglobulin are prepared, the F(ab')₂ fragments converted to Fab' fragments by reduction under mild reducing conditions, and then reoxidized in each other's presence under acidic conditions in accord with methods known *per se*. See also U.S. patent 4,444,878.

[0030] In an alternative method for producing a heterofunctional antibody, host cells producing an adhesion-immunoglobulin fusion, e.g. transfected myelomas, also are fused with B cells or hybridomas which secrete antibody having the desired companion specificity for an antigen. Heterobifunctional antibody is recovered from the culture medium of such hybridomas, and thus may be produced somewhat more conveniently than by conventional *in vitro* resorting methods (EP 68,763).

[0031] Another group of fusions are those in which an adhesion is conjugated with a toxic substance, e.g. a polypeptide such as ricin (including deglycosylated ricin A chain), diphtheria toxin A, or a non-peptidyl cytotoxin. Where the toxin is a polypeptide it is convenient to cross-link the polypeptide to the adhesion or its transmembrane-deleted variant by conventional *in vitro* protein cross-linking agents (for suitable methods for linking ricin A chain or deglycosylated A chain to CD4 see, for example, Duncan *et al.*, "Anal. Biochem." 132:68-73 [1983]; Thorpe *et al.*, "Cancer Res." 47: 5924 [1987]; and Ghotre *et al.*, "Cancer Res." 48:2610 [1988]) or by recombinant synthesis as a fusion (see for example, U.S. Patent 4,765,382). Alternatively, where companion antibodies are anti-ricin antibody immunoglobulin variable domains, such immunoglobulin heteroantibodies are employed to deliver ricin to HIV infected cells following the general procedure of Raso *et al.*, Cancer Research, 41:2073 (1981).

[0032] Another class of adhesion variants are deletional variants. Deletions are characterized by the removal of one or more amino acid residues from a adhesion sequence. Typically, the transmembrane and cytoplasmic domains of adhesions are deleted. In the case of CD4, at least residues 368 to 395 (the transmembrane region), and ordinarily 396-433 as well (the cytoplasmic domain), will be deleted to obtain secreted forms of this adhesion. Parenthetically, the amino acid residues follow the numbers given for mature CD4 as noted, for example, in figures 1a - 1c.

[0033] Substitutional variants are those in which at least one residue in the adhesion sequence has been removed and a different residue inserted in its place. The native N-terminal residue for mature CD4 is now known to be lysine. Thus, the sequence shown in Fig. 1, with an N-terminal asparagine, is an amino acid sequence variant of native mature CD4. Table 2 below describes substitutions which in general will result in fine modulation of the characteristics of the CD antigen.

TABLE 2

Original Residue	Exemplary Substitutions
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser; ala
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

[0034] Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in adhesion properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteinyl or prolyl is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanyl, is substituted for (or by) one not having a side chain, e.g., glycyl.

[0035] A preferred class of substitutional or deletional variants are those involving the transmembrane region of the adhesion. The transmembrane region of the adhesion is a highly hydrophobic or lipophilic domain that is the proper size to span the lipid bilayer of the cellular membrane. It is believed to anchor the adhesion in the cell membrane.

[0036] Deletion or substitution of the transmembrane domain will facilitate recovery and provide a soluble form of the adhesion by reducing its cellular or membrane lipid affinity and improving its water solubility. If the transmembrane and cytoplasmic domains are deleted one avoids the introduction of potentially immunogenic epitopes, either by exposure of otherwise intracellular polypeptides that might be recognized by the body as foreign or by insertion of heterologous polypeptides that are potentially immunogenic. A principal advantage of the transmembrane deleted adhesion is that it is secreted into the culture medium of recombinant hosts. This variant is water soluble and does not have an appreciable affinity for cell membrane lipids, thus considerably simplifying its recovery from recombinant cell culture.

[0037] It will be amply apparent from the foregoing discussion that substitutions, deletions, insertions or any combination thereof are introduced to arrive at a final construct. As a general proposition, all variants will not have a functional transmembrane domain and preferably will not have a functional cytoplasmic sequence. This is generally accomplished by deletion of the relevant domain, although adequate insertional or substitutional mutagens also can be effective for this purpose. For example, the transmembrane domain is substituted by any amino acid sequence, e.g. a random or homopolynucleic sequence of about 5 to 50 serine, threonine, lysine, arginine, glutamine, aspartic acid and like hydrophilic residues, which altogether exhibit a hydrophilic hydropathy profile, so that it is secreted into the culture medium

of recombinant hosts. This variant should also be considered to be an adhesion variant.

[0038] These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the adhesion, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant adhesions also are prepared by *in vitro* synthesis. Obviously, variations made in the DNA encoding the variant adhesions must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure deleterious to expression (EP 75,444A). The CD4 variants typically exhibit the same gp120 binding activity as does the naturally-occurring prototype, although variants also are selected in order to modify the characteristics of the CD4 adhesion as indicated above.

[0039] While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed adhesion variants screened for the optimal combination of desired activities. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis.

[0040] Adhesion variants that are not capable of binding HIV gp120 are useful nonetheless as immunogens for raising antibodies to the adhesion or as immunoassay kit components (labelled, as a competitive reagent for gp120 assay, or unlabelled as a standard for an adhesion assay) so long as at least one adhesion epitope remains active.

[0041] The DNA encoding adhesions is obtained by known procedures. See Reinhertz *et al.* and Maddon *et al.*, *op cit.* In general, prokaryotes are used for cloning of CD4 variant DNA sequences. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* B, UM101 and *E. coli* χ 1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

[0042] DNA encoding the variant adhesions are inserted for expression into vectors containing promoters and control sequences which are derived from species compatible with the intended host cell. The vector ordinarily, but need not, carry a replication site as well as one or more marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using a derivative of pBR322 which is a plasmid derived from an *E. coli* species (Bolivar, *et al.*, *Gene* 2: 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA constructions.

[0043] Promoters suitable for use with prokaryotic hosts illustratively include the β -lactamase and lactose promoter systems (Chang *et al.*, *Nature*, 275: 615 [1978]; and Goeddel *et al.*, *Nature* 281: 544 [1979]), alkaline phosphatase, the tryptophan (*trp*) promoter system (Goeddel, *Nucleic Acids Res.* 8: 4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the *tac* promoter (H. de Boer *et al.*, *Proc. Natl. Acad. Sci. USA* 80: 21-25 [1983]). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to DNA encoding the adhesion variant using linkers or adaptors to supply any required restriction sites (Siebenlist *et al.*, *Cell* 20: 269 [1980]). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antigen.

[0044] In addition to prokaryotes, eukaryotic microbes such as yeast cultures also are useful as cloning or expression hosts. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb, *et al.*, *Nature* 282: 39 [1979]; Kingsman *et al.*, *Gene* 7: 141 [1979]; Tschemper *et al.*, *Gene* 10: 157 [1980]) is commonly used. This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, *Genetics* 85: 12 [1977]). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective means of selection by growth in the absence of tryptophan.

[0045] Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.* 255: 2073 [1980]) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.* 7: 149 [1968]; and Holland, *Biochemistry* 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mu-tase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0046] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman *et al.*, European Patent Publication No. 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

[0047] Promoters are controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. the beta actin

promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, **273**: 113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *HindIII* E restriction fragment. Greenaway, P.J. *et al.*, *Gene* **18**: 355-360 (1982). Of course, promoters from the host cell or related species also are useful herein.

[0048] DNA transcription in higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually from about 10 to 300bp, that act to increase the transcription initiation capability of a promoter. Enhancers are relatively orientation and position independent having been found 5' (Laimins, L. *et al.*, *Proc.Natl.Acad.Sci.* **78**: 993 [1981]) and 3' (Lusky, M.L., *et al.*, *Mol. Cell Bio.* **3**: 1108 [1983]) to the transcription unit, within an intron (Banerji, J.L. *et al.*, *Cell* **33**: 729 [1983]) as well as within the coding sequence itself (Osborne, T.F., *et al.*, *Mol. Cell Bio.* **4**: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0049] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the adheson.

[0050] Expression vector systems generally will contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented medium. Two examples are: CHO DHFR⁻ cells and mouse LTK⁻ cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented medium. An alternative to supplementing the medium is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non supplemented media.

[0051] The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, Southern P. and Berg, P., *J. Molec. Appl. Genet.* **1**: 327 (1982), mycophenolic acid, Mulligan, R.C. and Berg, P. *Science* **209**: 1422 (1980) or hygromycin, Sugden, B. *et al.*, *Mol. Cell. Biol.* **5**: 410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

[0052] "Amplification" refers to the increase or replication of an isolated region within a cell's chromosomal DNA. Amplification is achieved using a selection agent e.g. methotrexate (MTX) which inactivates DHFR. Amplification or the making of successive copies of the DHFR gene results in greater amounts of DHFR being produced in the face of greater amounts of MTX. Amplification pressure is applied notwithstanding the presence of endogenous DHFR, by adding ever greater amounts of MTX to the media. Amplification of a desired gene can be achieved by cotransfecting a mammalian host cell with a plasmid having a DNA encoding a desired protein and the DHFR or amplification gene permitting cointegration. One ensures that the cell requires more DHFR, which requirement is met by replication of the selection gene, by selecting only for cells that can grow in the presence of ever-greater MTX concentration. So long as the gene encoding a desired heterologous protein has cointegrated with the selection gene replication of this gene gives rise to replication of the gene encoding the desired protein. The result is that increased copies of the gene, i.e. an amplified gene, encoding the desired heterologous protein express more of the desired heterologous protein.

[0053] Preferred host cells for expressing the CD antigen variants of this invention are mammalian cell lines, examples including: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, Graham, F.L. *et al.* *J. Gen Virol.* **36**: 59 [1977] and 293s cells [293 subclones selected for better suspension growth]); baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin, *Proc.Natl.Acad.Sci. (USA)* **77**: 4216, [1980]); mouse sertoli cells (TM4, Mather, J.P., *Biol. Reprod.* **23**: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51 cells); and TRI cells (Mather, J.P. *et al.*, *Annals N.Y. Acad. Sci.* **383**: 44-68 [1982]).

[0054] "Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extra-chromosomal element or by chromosomal integration. One suitable for transformation of the host cells is the method of Graham, F. and van der Eb, A., *Virology* **52**: 456-457 (1973). However, other methods for introducing DNA into cells

such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell walls are used as hosts, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F.N. et al., Proc. Natl. Acad. Sci. (USA), 69: 2110 (1972).

[0055] Construction of suitable vectors containing the desired coding and control sequences employ standard and manipulative ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required. Suitable procedures are well known for the construction described herein. See, for example, (Man iatis, T. et al., Molecular Cloning, 133-134 Cold Spring Harbor, [1982]; "Current Protocols in Molecular Biology", edited by Ausubel et al., [1987], pub. by Greene Publishing Associates & Wiley-Interscience).

[0056] Correct plasmid sequences are confirmed by transforming *E. coli* K12 strain 294 (ATCC 31446) with ligation mixtures, successful transformants selected by ampicillin or tetracycline resistance where appropriate, plasmids from the transformants prepared, and then analyzed by restriction enzyme digestion and/or sequenced by the method of Messing et al., Nucleic Acids Res. 9: 309 (1981) or by the method of Maxam et al., Methods in Enzymology 65: 499 (1980).

[0057] Host cells are transformed with the expression vectors of this invention. Thereafter they are cultured in appropriate culture media, e.g. containing substances for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0058] The secreted adhesion variants are recovered and purified from the culture supernatants of recombinant hosts. Typically, the supernatants are concentrated by ultrafiltration, contacted with a ligand affinity or immunoaffinity matrix so as to adsorb the adhesion variant, and eluted from the matrix. Optionally, the adhesion is purified by ion exchange chromatography.

[0059] Surprisingly, purification of soluble CD4 adhesion from culture medium was unexpectedly difficult. Notwithstanding that the hydrophobic transmembrane region of the antigen had been deleted, the antigen exhibited a strong tendency to form aggregates that could be readily removed from suspension by centrifugation at 1000 x g, and which avidly coat surfaces such as ultrafiltration membranes. This appears to result from the reduction in concentration of albumin or other serum protein (ordinarily present in the crude preparation) to a particular level, below which the truncated antigen no longer remains soluble. This phenomenon appears to be aggravated by exposure of the CD4 adhesion to low pH (< about pH 4). As a result, separation procedures (particularly those that employ acid elution, such as immunoaffinity) should be modified so that the eluate is maintained at, or immediately returned to, about neutrality. Further, a surfactant, e.g. a detergent such as Tween 80, should be included with the antigen during the separation procedure. The final purified product will be stabilized with a predetermined protein such as albumin, and/or a detergent.

[0060] The purified adhesion is formulated into conventional pharmacologically acceptable excipients.

[0061] It is administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of soluble CD4 adhesion/ml plasma. For CD4 adhesion variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma will be initially evaluated clinically in order to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor. The ordinary dosage of soluble CD4 is 100 µg/kg of patient weight/day.

[0062] The therapeutic CD4 variants are employed with other therapies and agents for the treatment of AIDS, including AZT, neutralizing antibodies and immunocytotoxins, gp120 fragments and vaccines.

[0063] In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

[0064] "Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

[0065] "Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

[0066] "Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally (Lawn, R. et al., Nucleic

Acids Res. 9: 6103-6114 [1981], and Goeddel, D. *et al.*, Nucleic Acids Res. 8: 4057 [1980]).

[0067] "Dephosphorylation" refers to the removal of the terminal 5' phosphates by treatment with bacterial alkaline phosphatase (BAP). This procedure prevents the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Procedures and reagents for dephosphorylation and other recombinant manipulations are conventional. Reactions using BAP are carried out in 50 mM Tris at 68°C to suppress the activity of any exonucleases which may be present in the enzyme preparations. Reactions were run for 1 hour. Following the reaction the DNA fragment is gel purified.

[0068] "Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T. *et al.*, *Id.* at 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

[0069] "Filling" or "blunting" refers to the procedures by which the single stranded end in the cohesive terminus of a restriction enzyme-cleaved nucleic acid is converted to a double strand. This eliminates the cohesive terminus and forms a blunt end. This process is a versatile tool for converting a restriction cut end that may be cohesive with the ends created by only one or a few other restriction enzymes into a terminus compatible with any blunt-cutting restriction endonuclease or other filled cohesive terminus. Typically, blunting is accomplished by incubating 2-15µg of the target DNA in 10mM MgCl₂, 1mM dithiothreitol, 50mM NaCl, 10mM Tris (pH 7.5) buffer at about 37°C in the presence of 8 units of the Klenow fragment of DNA polymerase I and 250 µM of each of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. phenol and chloroform extraction and ethanol precipitation.

[0070] The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention. All literature citations herein are expressly incorporated by reference.

Example 1

Construction of Vectors for the Expression of Native CD4 and Secreted Derivatives

Section 1

[0071] The plasmid used for recombinant synthesis of human CD4 was pSveCD4DHFR. The plasmid was constructed as follows:

[0072] λCD4P1 containing most of the coding sequence of human CD4 (obtained from a human placental cDNA library using oligonucleotide probes based on the published sequence [Maddon *et al.* 1985]) was digested with EcoRI to produce the cDNA insert. This fragment was recovered by polyacrylamide gel electrophoresis (fragment 1).

[0073] pUC18 was digested with EcoRI and the single fragment recovered by polyacrylamide gel electrophoresis (fragment 2). Fragment 1 was ligated to fragment 2 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct DNA fragments. This plasmid is referred to as pUCCD4.

[0074] pSveE'DHFR (Muesing *et al.*, Cell 48:691-701 [1987]) was digested with KpnI and BamHI and blunted with E. coli DNA polymerase I (Klenow fragment) and the four dNTPs. Fragment 3 containing the pML-Amp^r region, SV40 early promoter, the HIV LTR, and the mouse DHFR gene was recovered by gel electrophoresis, ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the BamHI restriction site and the absence of the KpnI restriction site. This plasmid is referred to as pSveΔBKDHFR and allows EcoRI-BamHI fragments to be inserted after the SV40 early promoter and transcribed under its control, following transfection into an appropriate cell line.

[0075] Synthetic oligonucleotides (adaptors 1-8, below) were made to extend from 76 bp 5' of the initiation codon of CD4 translation to the RsaI restriction site at 121 bp 3' of the initiator, with the sequence AATT at the 5' end of the sense strand to generate an end which could ligate to an EcoRI restriction fragment. These oligonucleotides were ligated and the 204 bp fragment containing the entire sequence recovered by gel electrophoresis (fragment 4).

CD4 adaptor 1: AATTC AAGCCCAGAGCCCTGCCATTCTGTGGGCTCAGGTCCT

CD4 adaptor 2: pACTCCTCAGCCCCCTTCCTCCCTCGGCAAGGCCACAATGAACCGGGGAGTC

CD4 adaptor 3: pCCTTTTAGGCACTTGCTTCTGCTGCTGCAACTGGGGCTCCTCCCAGC

CD4 adaptor 4:

pAGCCACTCAGGGAAACAAAGTGGTGGTGGGCAAAAAAGGGGATACAGTGGAACTGACCTGT

CD4 adaptor 5: pACAGGTCAGTTGCACTGTATCCCCTTTTTTGGCCAGCACCCACTTTGTTTC

CD4 adaptor 6: pCTGAGTGGCTGCTGGGAGGAGCGCCAGTTGCAGCACCAGAAGCAAGT

CD4 adaptor 7: pGCCTAAAAGGGACTCCCGCGTTTCATTGTGGCCTTGCCGAGCGACGAAGCG

CD4 adaptor 8: GCTGAGCAGTAGGGACCTGAGCCCACAGAAATGGCAGGGCTCTGGGCTTG

[0076] pUCCD4 was digested with RsaI and SstI and the 401 bp fragment containing part of the CD4 coding sequence recovered by gel electrophoresis (fragment 5). pUC18 was digested with EcoRI and SstI and the fragment comprising the bulk of the plasmid recovered by gel electrophoresis (fragment 6). Fragments 4 and 5 were ligated to fragment 6 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of the inserted synthetic DNA was checked by excising the 605 bp EcoRI-SstI fragments from several transformants and ligating them to M13mp19 which had been digested with the same enzymes. After transformation into *E. coli* strain JM101, single-stranded DNA was prepared and sequenced. One plasmid which contained the correct sequence was selected, and is referred to as pCD4int.

[0077] pCD4int was digested with EcoRI and SstI and fragment 7 containing the 5' end of the CD4 coding region was recovered by gel electrophoresis. pUCCD4 was digested with SstI and BamHI and the 1139 bp fragment containing the remainder of the CD4 coding region (fragment 8) recovered by gel electrophoresis.

[0078] pSveΔBKDHFR was digested with EcoRI and BamHI and fragment 9 comprising the bulk of the plasmid was isolated. Fragments 7, 8 and 9 were ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and the resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pSveCD4DHFR, and was used to direct synthesis of recombinant intact CD4.

Section 2

[0079] A plasmid was constructed to direct the synthesis of a CD4 derivative lacking the putative transmembrane domain and most of the putative cytoplasmic domain (Maddon *et al.*). This was done with the intention of creating a secreted form of CD4, based on the assumption that these domains anchor the CD4 glycoprotein to the cell membrane, and that their deletion would result in the secretion of the product. This plasmid is referred to as pSveCD4ΔN1aDHFR and was constructed as follows:

[0080] pUCCD4 was digested with SstI and TaqI and the 531 bp fragment (fragment 10) recovered. pUCCD4 was digested with NlaIII and TaqI and the 112 bp fragment (fragment 11) recovered. pUCCD4 was digested with BamHI and NlaIII and the 301 bp fragment (fragment 12) recovered. pCD4int was digested with SstI and BamHI and fragment 13 comprising the bulk of the plasmid recovered. Fragments 10, 11, and 12 were ligated together with fragment 13 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. Plasmid DNA from several transformants was sequenced to ensure that the 195 bp NlaIII fragment had been deleted and that the proper reading frame was restored. The resulting plasmid is referred to as pCD4ΔN1a.

[0081] pCD4ΔN1a was digested with EcoRI and BamHI and the 1541 bp fragment containing the sequence of a CD4 derivative lacking the transmembrane and cytoplasmic domains recovered (fragment 14) and ligated to fragment 9 and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction

analysis for the presence of the correct fragment. This plasmid is referred to as pSVeCD4ΔN1aDHFR.

[0082] Both pSVeCD4DHFR and pSVeCD4ΔN1aDHFR were transfected into CHO cells by the same method used to establish cell lines stably expressing HIV-I polypeptides (Muesing, Smith and Capon, Cell 48:6910701 [1987]). These cells were assayed for production by radioimmunoprecipitation as described below. While no product was detected in initial experiments, subsequent experiments showed that the above described coding segment could indeed direct the synthesis of a soluble CD4 adhesion variant both in CHO and 293 cells.

Section 3

[0083] A different expression system was initially used for the synthesis and expression of a CD4 variant lacking completely the cytoplasmic and transmembrane domains. The system uses the cytomegalovirus promoter and can be used in cultured cells of human origin. The first plasmid constructed for use in this system contained the entire coding region for CD4 and was intended to function as a control in the following studies. It is referred to as pRKCD4, and was constructed as follows:

[0084] pSVeCD4DHFR was digested with EcoRI and BamHI and fragment 15 containing the entire CD4 coding region was isolated. pRK5 (European Application No. 88308386.7) was digested with EcoRI and BamHI and fragment 16 comprising the bulk of the plasmid recovered by gel electrophoresis, ligated to fragment 15, and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4.

Section 4

[0085] The next plasmid constructed was designed to direct the expression of the above-mentioned (Section 3) secreted derivative of CD4. The coding region of CD4 was fused after amino acid residue 368 of mature CD4 to a sequence from pBR322 which codes for 9 more residues before a translation termination codon. This removes the putative CD4 transmembrane and cytoplasmic domains, which are presumed to anchor CD4 to the cell surface. The plasmid is referred to as pRKCD4T, and was constructed as follows:

[0086] pSVeCD4DHFR was digested with HpaII, blunted with Klenow fragment and the four dNTPs, and digested with BstEII. The 382 bp fragment (fragment 17) containing part of the CD4 coding sequence was recovered by gel electrophoresis. pSVeCD4DHFR was digested with EcoRI and BstEII and the 874 bp fragment (fragment 18) recovered. pBR322 was digested with HindIII, blunted with Klenow fragment and the four dNTPs, and digested with EcoRI. Fragment 19 comprising the bulk of the plasmid was isolated and ligated to fragments 17 and 18 and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pCD4Tint.

[0087] pRK5 was digested with EcoRI and SmaI and fragment 20 comprising the bulk of the plasmid isolated. pCD4Tint was digested with EcoRI and EcoRV and the 1410 bp fragment containing the CD4 coding sequence to the HpaII site at 1176 bp 3' of the initiating codon and the 154 bp HindIII-EcoRV fragment of pBR322 was recovered (fragment 21). Fragments 20 and 21 were ligated and the ligation mixture transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. This plasmid is referred to as pRKCD4T.

Section 5a

[0088] In order to create a secreted form of CD4 which could be purified with an antibody directed to herpes virus type I glycoprotein D, a plasmid was constructed to express a derivative of CD4T in which the region coding for the mature, processed CD4T polypeptide was fused to a sequence coding for the signal peptide and the first 27 residues of the mature type I Herpes Simplex Virus gD glycoprotein. This plasmid is referred to as pRKGD4T, and was constructed as follows:

[0089] pgDTrunc.DHFR was digested with EcoRI and PvuII and the fragment containing the coding region for the signal peptide and first 27 residues of the mature HSV I gD glycoprotein was isolated (fragment 22). pRKCD4T was digested with EcoRI and BstEII and fragment 23 containing the 3' end of the CD4 coding sequence and the pRK5 region was isolated.

[0090] Synthetic oligonucleotides GD (adaptors 1-2, below) containing the coding sequence of CD4 from the codon for the amino terminal residue of mature CD4 to the Rsa site at 121 bp 3' of translation initiation, and containing the sequence CTGCTCGAG at the 5' end of the sense strand were prepared (fragment 24). pRKCD4 was digested with

RsaI and BstEII and the 665 bp fragment containing part of the coding region for CD4 was recovered (fragment 25) and ligated to fragment 24. After digestion with BstEII to ensure that only monomeric fragment was present, the 724 bp fragment containing both sequences was recovered by gel electrophoresis (fragment 26).

[0091] Fragments 22, 23 and 26 were ligated and the ligation mixture transformed into *E. coli* strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The sequence of several transformants was checked to ensure that the synthetic insert was correct and that reading frame was preserved. This plasmid is referred to as pRKGD4T.

[0092] These pRK5 derived plasmids preferably were transfected into 293S cells for stable expression according to Muesing, et al. Cell 48:691 (1987) with the exception that in addition to the plasmid of interest a plasmid expressing the neomycin resistance gene pRSV neo (Gorman et al. Science 221:553-555 (1985)) was cotransfected. 293 cells also are used satisfactorily as host cells. 2 days after transfection, the cells were passaged into standard medium (1:1 F12/DME supplemented with L-glutamine, penicillin-streptomycin and 10% FBS) with 0.5 mg/ml G418 (Genticin sulfate; Gibco) for selection of stable cell lines, rather than in media containing methotrexate as shown by Muesing et al. Cells were assayed for production of CD4 or CD4 analogs by radioimmunoprecipitation. Binding studies (section 5c) used conditioned supernatants from these cells in the 1:1 F12/DME medium. Materials used in infectivity assays (section 5b) were obtained as described in section 8 below.

gDCD4 adaptor 1:

CTGCTCGAGCAGGGAAACAAAGTGGTGCTGGGCCAAAAAGGGGATACAGTGGAACTGAC

gDCD4 adaptor 2:

PACAGGTCAGTTCCACTGTATCCCTTTTTTGCCAGCACCACCTTTGTTTCCCTGCTCGA

Section 5b

[0093] The following constitutes a study of the neutralization of HIV-1 infectivity by soluble CD4 analogs. A modification of the neutralization procedure of Robert-Guroff et al., Nature 316:72 (1985) was followed. Equal volumes of inhibitor supernatant and virus (60 microliters) were incubated at 4 degrees C for 1 hour, then the same volume of H9 (Gallo et al., Science 224:500, 1984) at 5×10^6 /ml was added and incubation continued for 1 hour at 37 degrees C. Following absorption, 2.5×10^5 cells in 150 microliters were transferred to 2 ml of incubation media. After 4 days at 37 degrees C, the cultures were split 1:2 with fresh media and incubated for an additional 3 days. Cultures were harvested, reverse transcriptase activity was measured (Groopman et al., AIDS Research and Human Retroviruses 3:71, 1987), and immunofluorescence reactivity with HIV-1 positive serum was determined as described (Poesz et al., Proc. Acad. Nat. Sci. USA 77:7415, 1980). Inhibitor supernatants were obtained from confluent plate cultures of 293s/CDT4, 293s/gDCD4T cells or untransfected 293s cells by replacing the growth medium incubation media and harvesting the supernatants 24 hours later. Inhibitor supernatant replaced part or all of the incubation media during the first three days of culture as indicated in the second column of Table 3. Challenge dose of virus was 100 TCID₅₀ (Groopman et al., supra) of HIV-1 strain HTLV-IIIb grown in H9 cells assayed in the same system. Incubation media consisted of RPMI 1640 media containing 2mM L-glutamine, 100 units/ml penicillin, 100 micrograms/ml streptomycin, 2 micrograms/ml polybrene and 20% fetal calf serum (M.A. Bioproducts).

Table 3

Inhibitor supernatant	Dilution of Inhibitor supernatant	Indirect immunofluorescence (% positive cells)		Reverse transcriptase (cpm/ml x 10 ⁵)	
mock-transfected	undil.; 1:4	65.3	65.5	21.8	23.9

Table 3 (continued)

Inhibitor supernatant	Dilution of Inhibitor supernatant	Indirect immuno- positive		Reverse transcr 10	
mock-transfected	undil.; 1:4	61.2	61.1	18.5	28.1
CD4T	undil.; 1:4	0.4	18.0	0.11	5.94
CD4T	undil.; 1:4	0.8	16.1	0.15	3.72
gDCD4T	undil.; 1:4	0.4	26.8	0.14	9.92
gDCD4T	undil.; 1:4	1.4	36.1	0.23	11.3

[0094] Both forms of soluble CD4 virtually abolished the growth of HIV-1, when incubated with virus-infected cells without prior dilution (Table 2). At a dilution of 1:4 the soluble CD4 preparations were only partially effective in inhibiting virus growth, however the level of fluorescent-positive cells and reverse transcriptase was still significantly lower than cultures receiving mock-transfected cell supernatants (Table 2). Since there was no significant difference in virus growth between diluted and undiluted control supernatants, nor did any of the supernatants affect the growth of uninfected H9 cells (data not shown), soluble CD4 proteins present in these supernatants were concluded to be responsible for the neutralization of HIV-1 infection of H9 cells.

Section 5c

[0095] To determine the affinity constant for interactions between gp120 and CD4 or CD4 variants, saturation binding analysis was carried out with soluble CD4 (supra) and detergent solubilized intact CD4 (Lasky et al. Cell 50:975 [1987]) employing radioiodinated gp120 labeled with lactoperoxidase. Binding reactions consisted of 125 I-gp120 (3 ng to 670 ng, 2.9 nCi/ng) incubated for 1 hour at 0 degrees C with cell lysates containing intact CD4 (Laskey et al., op cit.) or cell supernatants containing unlabeled CD4T or gDCD4T prepared as described in section 5a. Reactions (0.2ml) had a final composition of 0.5X McDougal Lysis Buffer (McDLB) (1 x McDLB contains 0.5 % Nonidet NP-40, 0.2% Na deoxycholate, 0.12 M NaCl, 0.02 M Tris-HCl, pH 8.0) and were performed in duplicate, both in the presence or absence of 50 micrograms of unlabeled purified gp120 (74 fold or greater excess). Following incubation, bound gp120 was quantitated by immunoprecipitation and counted in a gamma counter. For immunoprecipitation, binding reaction solutions were preabsorbed with 5 microliters of normal rabbit serum for one hour at 0°C, and cleared with 40 microliters of Pansorbin (10 % w/v, Calbiochem) for 30 minutes at 0 degrees C. Samples were then incubated overnight at 0 degrees C with 2 microliters of normal serum or 5 microliters (0.25 microgram) of OKT4 monoclonal antibody (Ortho) followed by collection of immune complexes with 10 microliters of Pansorbin. Precipitates were washed twice in 1X McDLB and once in water, then eluted by eluting at 100 degrees C for 2 minutes in sample buffer (0.12 M Tris-HCl pH 6.8, 4% SDS, 0.7 M mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue). CD4 molecules were bound saturably by gp120, and yielded a simple mass action binding curve. Supernatants from mock-transfected cells gave a level of specifically bound gp120 less than 1% that found for supernatants containing soluble CD4. Scatchard analysis revealed a single class of binding sites on each molecule, with apparent dissociation constants (K_d) of 1.3×10^{-9} M, 0.83×10^{-9} M and 0.72×10^{-9} M for intact CD4, CD4T and gDCD4T, respectively. The values obtained for CD4-gp120 binding in solution are comparable to the affinity previously measured for gp120 binding to CD4 on whole cells ($K_d=4.0 \times 10^{-9}$ M. Lasky, Cell, supra).

Section 6

[0096] In order to produce secreted derivatives of CD4 which are free of extraneous amino acid residues, two plasmids were constructed for expression in 293 cells. The plasmids contain CD4 genes which have been truncated without the addition of extra residues, and are referred to as pRKCD4 Δ N1a and pRKCD4TP, and were constructed as follows:

[0097] Fragment 14 containing the CD4 gene with the 195 bp N1aIII restriction fragment deleted was ligated to fragment 16, which is pRK5 digested with EcoRI and BamHI. The ligation mixture was transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4 Δ N1a.

[0098] Synthetic DNA was made to attach to the HpaII site at 1176bp and which when so attached would terminate translation after amino acid residue 370 of mature CD4 (fragment 27). The other end of this fragment was designed to ligate to BamHI restriction fragments. pUCCD4 was digested with BstEII and HpaII and the 382bp fragment containing

part of the CD4 gene was recovered (fragment 28). Fragments 27 and 28 were ligated and then digested with BstEII to reduce dimerized fragments to monomers, and the resulting 401bp fragment was recovered (fragment 29).

[0099] pRKCD4 was digested with BstII and BamHI and the fragment comprising the bulk of the plasmid (fragment 30) was isolated and ligated to fragment 29. The ligation mixture was transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4TP. Both plasmids are transfected into 293 cells to generate stable variant CD4-expressing cell lines as described above.

Section 7

[0100] Two plasmids were constructed to direct the expression of secreted CD4 lacking extraneous amino acid residues in CHO cells. These are referred to as pSveCD4ΔN1aSVDHFR and pSveCD4TPSVDHFR, and were constructed as follows:

[0101] pE34BHBV.E400D22 was digested with PvuI and EcoRI and the fragment containing the SV40 early promoter and part of the β-lactamase gene was recovered (fragment 31). pE348HBV.E400D22 was digested with PvuI and BamHI and the large fragment containing the balance of the β-lactamase gene as well as the SV40 early promoter and the DHFR gene was isolated (fragment 32).

[0102] Fragments 31 and 32 were ligated together with fragment 14 and transformed into E. coli strain 294. The transformed culture was plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pSVECD4ΔN1aSVDHFR. This plasmid contains the same DNA fragment encoding the soluble CD4 molecule found in the above-mentioned plasmid pSveCD4ΔN1aDHFR (Section 2).

[0103] pRKCD4TP was digested with EcoRI and BamHI and the fragment containing the truncated CD4 coding region was isolated and ligated to fragments 31 and 32. The ligation mixture was transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pSveCD4TPSVDHFR. Both of these plasmids are transfected into CHO cells and amplified transfectants selected by methotrexate using conventional procedures.

Example 2

[0104] Fusions of the V region of the CD4 gene, which is homologous to the variable region of immunoglobulin genes (ref Maddon *et al.* 1985), to the constant (C) region of human immunoglobulin κ and γ2 chains are constructed as follows:

[0105] Synthetic DNA is made to code for the C region of human κ chain (residues 109-214) based on the sequence published by Morin *et al.*, Proc. Natl. Acad. Sci. 82:7025-7029, with the addition at the 5' end of the coding strand of the sequence GGGG, which allows this fragment to be ligated to the BspMI site at the end of the putative V-like region of CD4. At the 3' end of the coding region, a translational stop codon is added as well as a sequence which allows this end to be ligated to BamHI restriction fragments. The synthetic DNA is made in 8 fragments, 4 for each strand, 70-90 bases long. These are then allowed to anneal and ligated prior to isolation on a polyacrylamide gel (fragment 33).

[0106] pRKCD4 is digested with EcoRI and BspMI and the 478bp fragment containing the region coding for the putative V-like domain of CD4 is recovered (fragment 34). Fragments 33 and 34 are ligated together with fragment 16 (from the expression vector pRK5). The ligation mixture is transformed into E. coli strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA is prepared from transformants and checked by restriction analysis for the presence of the correct fragment. The resulting plasmid is referred to as pRKCD4Ck.

[0107] A plasmid encoding a fusion of the CD4 V-like domain to the human immunoglobulin Cγ2 region is constructed in a similar fashion, and is referred to as pRKCD4Cγ2. Both of these plasmids are transfected into 293 cells, myeloma cells or other competent cells in order to obtain cell lines expressing variant CD4 molecules as described above.

Expression in CHO Cells

[0108] Plasmids were constructed to direct the expression of the immunoadhesons described above in CHO cells. These are referred to as pSveCD4_{4γ1}SVDHFR, pSveCD4_{2γ1}SVDHFR, pSveCD4_{e4γ1}SVDHFR, pSveCD4_{e2γ1}SVDHFR, pSveCD4_{4κ}SVDHFR and pSveCD4_{2κ}SVDHFR.

[0109] Fragment 31 was prepared as described above. Fragment 32a was prepared by digesting plasmid pE348HBV.E400 D22 with BamHI, blunting with Klenow fragment and the four dNTPs, then digesting with PvuI. Plasmids pRKCD4_{4γ1}, pRKCD4_{2γ1}, pRKCD4_{e4γ1}, pRKCD4_{e2γ1}, pRKCD4_{4κ} and pRKCD4_{2κ} were separately digested with HindIII,

blunted with Klenow fragment and the four dNTPs, then digested with *EcoRI*. The resulting DNA fragments were ligated together with fragments 31 and 32a and transformed into *E. coli* strain 294. Colonies were selected and checked for the presence of the correct plasmid as above, then transfected into CHO cells and amplified by methotrexate selection using conventional procedures.

Example 3

[0110] The gDCD4T secreted by the method of Example 1 was purified from cell culture fluid containing either 10% FBS (fetal bovine serum) or no added FBS. The conditioned cell culture fluid was first concentrated by ultrafiltration then purified by immunoaffinity chromatography. The immunoaffinity column was produced by coupling murine monoclonal antibody 5B6 (whose epitope is on the HSV-1 gD portion of the gDCD4T molecule) to glyceryl coated controlled pore glass by the method of Roy *et al.* 1984. The concentrated cell culture fluid is applied directly to the column and the contaminating proteins are washed away with neutral pH buffer. The column is then washed with neutral buffer containing tetramethylammonium chloride followed by neutral buffer containing Tween 80. The bound gDCD4T is eluted from the column with buffer at pH3 containing Tween 80 (0.1% w/v) and is neutralized immediately as it is eluted. The eluted neutralized gDCD4T is then concentrated by ultrafiltration and dialyzed/diafiltered to exchange the buffer for a physiological salt solution containing Tween 80 at approximately 0.1% w/v.

[0111] If the detergent is not present the gDCD4T forms aggregates as evidenced by the ability of centrifugation at approximately 10,000 Xg for 2 minutes to remove the gDCD4T from the solution. Incubation of gDCD4T at 4°C in 0.1M sodium acetate, 0.5M NaCl and 0.25M tris at pH 7 together with BSA, Tween 80 or glycerol as candidate stabilizers showed that, in the absence of a stabilizer the gDCD4T gradually aggregated over the space of 12 days to the point where only about 60-70% of the protein was soluble. However, use of 0.1% w/v Tween 80 or (0.5 mg/ml BSA ensured that about 100% or 80%, respectively, of the gDCD4T remained soluble over this period. Surprisingly glycerol was ineffective as a stabilizer and produced results inferior even to the control-at 8 days about 80% of the gDCD4T was aggregated when stored in the presence of glycerol.

Example 4

[0112] Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin $\gamma 1$. These plasmids are referred to as pRKCD4_{2 γ 1}, pRKCD4_{e4 γ 1}, pRKCD4_{2 γ 1}, pRKCD4_{e2 γ 1}, pRKCD4_{1 γ 1}, and pRKCD4_{e1 γ 1}.

[0113] Plasmid pRKCD4_{4 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0114] Plasmid pRKCD4_{e4 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0115] Plasmid pRKCD4_{2 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for glutamine residue 180 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0116] Plasmid pRKCD4_{e2 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 177 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0117] Plasmid pRKCD4_{1 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for aspartic acid residue 105 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0118] Plasmid pRKCD4_{e1 γ 1} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for leucine residue 100 of the mature CD4 polypeptide, immediately followed by the sequence coding for the constant region of human immunoglobulin $\gamma 1$, starting at the codon for serine residue 114 of mature human immunoglobulin $\gamma 1$ (Kabat *et al.*).

[0119] Construction of these plasmids required the prior construction of plasmid pRKCD4TP/ $\gamma 1$. It was constructed as follows:

[0120] A cDNA clone coding for human immunoglobulin $\gamma 1$ was obtained from a human spleen cDNA library (Clontech

Laboratories, Inc.) using oligonucleotides based on the published sequence (Ellison *et al.*, "Nucl. Acids Res." 10: 4071-4079 [1982]), and an EcoRI-EagI fragment (the EcoRI site was contributed by a linker) containing part of the variable and all of the constant region was obtained. This fragment was blunted with Klenow fragment, and recovered by gel electrophoresis (Fragment a1).

[0121] Plasmid pRKCD4TP was digested with XbaI and treated with Klenow Enzyme, and Fragment a2, containing the linearized plasmid was recovered by gel electrophoresis, and ligated with fragment a1. The ligation mixture was transformed into *E. coli* strain 294, the transformed culture plated on ampicillin media plates and resistant colonies selected. Plasmid DNA was prepared from the transformants and checked by restriction analysis for the presence of the correct fragment in the correct orientation (i.e., the immunoglobulin coding region in the same orientation as the CD4 coding region, and at the 3' end of the CD4 coding region). This plasmid is referred to as pRKCD4TP/ γ 1.

[0122] Synthetic oligonucleotides were made as primers for deletional mutagenesis reactions to fuse the appropriate coding sequences of IgG1 and CD4 as described above. These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH₂-terminal 8 residues of the desired immunoglobulin moiety). Plasmid pRKCD4TP/ γ 1 was transformed into *E. coli* strain SR101 and the transformed cultures plated on ampicillin media plates. Resistant colonies were selected and grown in the presence of m13KO7 bacteriophage to yield secreted, encapsidated single-stranded templates of pRKCD4TP/ γ 1. The single-stranded plasmid DNA was isolated and used as the template for mutagenesis reactions with the synthetic oligonucleotides described above as primers. The mutagenesis reactions were transformed *E. coli* SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (ref. Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13KO7 bacteriophage. Templates were prepared as above and screened by sequencing.

[0123] The plasmids were transfected into 293 cells using standard procedures and assayed for expression and production as described above.

	Expressed	Secreted
pRKCD4 _{e1} γ 1	-	-
pRKCD4 ₁ γ 1	+	-
pRKCD4 _{e2} γ 1	+	+
pRKCD4 ₂ γ 1	+	+
pRKCD4 _{e4} γ 1	+	+
pRKCD4 ₄ γ 1	+	+

[0124] Plasmids also were constructed to direct the expression of fusion proteins containing differing lengths of the amino-terminal, extracellular domain of CD4 fused to the truncated portion of the constant region of human immunoglobulin γ 1, comprising only the hinge region and constant domains CH₂ and CH₃.

[0125] Synthetic oligonucleotides were made as primers for mutagenesis reactions to delete the immunoglobulin sequence from Ser114 to Cys215 inclusive (Kabat *et al.*). These were synthesized as 48-mers comprising 24 nucleotides on each side of the desired fusion site (i.e., corresponding to the COOH-terminal 8 residues of the desired CD4 moiety, and the NH₂-terminal 8 residues of the desired immunoglobulin moiety). Plasmids pRKCD4₄ γ 1, pRKCD4₂ γ 1 and pRKCD4₁ γ 1 were separately transformed into *E. coli* strain SR101 and the transformed culture plated on ampicillin media plates. Transformants were screened by colony hybridization (Grunstein-Hogness) for the presence of the appropriate fusion site, using 16mers as probes. These 16mers comprise 8 bases on either side of the fusion site, and the hybridization conditions chosen were sufficiently stringent that the probes only detect the correctly fused product. Colonies identified as positive were selected and plasmid DNA was isolated and transformed into *E. coli* strain SR101. The transformed cultures were plated on ampicillin media plates, and resistant colonies were selected and grown in the presence of m13KO7 bacteriophage. Templates were prepared as above and screened by sequencing.

[0126] The plasmid derived from plasmid pRKCD4₄ γ 1 is referred to as pRKCD4_{4Fc1}, that derived from plasmid pRKCD4₂ γ 1 is referred to as pRKCD4_{2Fc1} and that derived from plasmid pRKCD4₁ γ 1 is referred to as pRKCD4_{1Fc1}.

[0127] pRKCD4_{2Fc1}, pRKCD4_{1Fc1} and pRKCD4_{4Fc1} are cultured in the same fashion as described above and CH1-deleted CD4 immunoadhesions recovered as described elsewhere herein.

Light Chain Fusions

[0128] Plasmids were constructed to direct the expression of proteins containing differing lengths of the amino terminal, extracellular domain of CD4 fused to the constant region of human immunoglobulin κ . These plasmids are referred to as pRKCD4_{4 κ} and pRKCD4_{e4 κ} .

[0129] Plasmid pRKCD4_{4 κ} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for serine residue 366 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin κ , starting at the codon for threonine residue 109 of the mature human immunoglobulin κ . (Kabat *et al.*).

[0130] Plasmid pRKCD4_{e4 κ} contains the portion of the CD4 gene from the initiation codon to the fusion site after the codon for lysine residue 360 of the mature CD4 polypeptide, immediately followed by the sequence for the constant region of human immunoglobulin κ , starting at the codon for threonine residue 109 of the mature human immunoglobulin κ (Kabat *et al.*)

[0131] These plasmids were constructed in a manner analogous to plasmid pRKCD4_{4 γ} described above, with the following exception:

[0132] The human immunoglobulin κ coding sequence (Fig. 5) was obtained from a human spleen cDNA library (Clontech Laboratories, Inc.) using oligonucleotides based on the published sequence (Hieter, P.A. *et al.*, Cell 22:197-207 [1980]) and an *EcoRI*-*Bsp*MI fragment containing part of the variable region and the entire constant region was obtained. This fragment was blunted with Klenow fragment and the four dNTPs. This fragment was used instead of fragment al, and was used to construct plasmid pRKCD4TP/h κ .

Example 5Culture, Purification and formulation of CD4 variants

[0133] Plasmids encoding CD4T, prolyl terminal (CD4TP), or CD4T immunoadhesons were calcium phosphate transfected into CHO-DP7 (a proinsulin-transformed autocrine host cell derived from CHO; U.S.S.N. 97,472) and the transformants grown in selective medium (1:1 HAM F12/DMEM GHT⁻ containing 1 - 10% dialyzed or dialyzed bovine serum). Other suitable host cells are CHO cells or 293s human embryonic kidney cells. The transformants were subcloned into the same medium but containing 500 nm methotrexate. A subclone capable of secreting CD4T, CD4tp 500 b, was selected. CD4tp 500 b is cultured in a DMEM/HAM F12 medium at about 37°C until CD4T accumulates in the culture, after which the medium is separated from the cells and insoluble matter by centrifuging.

[0134] Culture fluid from CD4TP transformants was concentrated and dialyzed to lower the ionic strength. The concentrate was passed through a large volume of Q-Sepharose anion exchange resin (previously equilibrated with 25 mM NaCl, pH 8.5) in order to adsorb contaminants from the culture fluid. The isoelectric point of CD4TP is about 9.5, thus making it possible to discriminate between truncated forms of CD4 and most contaminants by alternate adsorption, respectively, on a cation exchange resin such as carboxymethyl or sulfonyl Sepharose, and an anion exchange resin such as a quaternary ammonium Sepharose. In addition, since highly electropositive domains are present in the extracellular segment of CD4 any CD4-containing variant is purified in the same fashion as CD4TP. The unadsorbed culture fluid from the anion exchange resin step was then passed through a cation exchange resin (previously equilibrated with 25 mM NaCl at pH 8.5) whereby CD4TP was adsorbed to the resin. The CD4TP was eluted with a NaCl gradient at pH 8.5, this CD variant eluting at about 0.2 mM NaCl. Ammonium sulfate was added to the eluate to the concentration of 1.7M and the solution passed through a column of hydrophobic interaction chromatography resin (phenyl or butyl Sepharose). The CD4TP was eluted from the hydrophobic interaction column with a gradient of ammonium sulfate, the CD4TP emerging at about 0.7M ammonium sulfate. The eluate was concentrated and buffer exchanged on a G-25 column using phosphate buffered saline containing .02 % (w/v) Tween 20 or Tween 80. The CD4TP was soluble and stable in this solution, which was sterile filtered and filled into vials as an aqueous formulation. Other polymeric nonionic surfactants are suitably used with the CD4 formulations, including Pluronic block copolymers or polyethylene glycol.

[0135] It is also possible to employ immunoaffinity purification of soluble CD4 wherein the CD4 is adsorbed onto an immobilized antibody against CD4. This method suffers from the disadvantage that elution of the CD4T under acidic conditions leads to protein aggregation that is only thoroughly ameliorated at relatively higher levels of surfactant. The foregoing procedure permits the use of much lower quantities of surfactant, about from 0.01 to 0.10 % (w/v) surfactant.

[0136] The procedure followed for the purification of CD4 fusions with immunoglobulin heavy chain was to concentrate recombinant supernatants by ultrafiltration and thereafter adsorb the fusion onto resin-immobilized Staphylococcal protein A. The fusion was eluted with 0.1M citrate buffer pH 3 with no salt or detergent. This preparation is buffered into tris buffer at pH 7.5. The immunoglobulin fusions with CD4 V1-V4 optionally are further purified by the procedure described above for unfused CD4 variants. CD4 immunoglobulin fusions with CD4 V1-V2 also may be purified by the

procedure above, except that it is not expected that the isoelectric point of this class of molecules will be as alkaline as that of species containing all four V regions of CD4.

Example 6

[0137] The characteristics of several adhesion variants were determined. As shown in table 4 the immunoadhesons CD4_{4γ1} and CD4_{2γ1} show improved plasma half-life in rabbits, coupled with high-affinity gp120 binding and an affinity for Fcγ receptor (determined with U937 cells) that is comparable to that of bulk human IgG.

Table 4

	gp120 KD (nM) [#]	FcγR KD (nM) ⁺	Plasma Half-Life ⁺⁺ In Rabbits (Hrs.)
CD4T [§]	2.3 ± 0.4	-	0.25
CD4 _{4γ1}	1.2 ± 0.1	2.83 ± 0.25	6.4
CD4 _{2γ1}	1.4 ± 0.1	3.01 ± 0.68	40.6
human IgG	ND	3.52 ± 0.5	21 days*

* determined in humans

⁺ KD was determined by the method of Anderson *et al.*, "J. Immunol." 125:2735-2741 (1980).

[#] determined by the method of Smith *et al.*, "Science" 238:1704-07 (1987).

[§] residues 1-368 only

⁺⁺ The adhesion variant was injected intravenously into rabbits and samples of blood were collected periodically and assayed for the presence of the adhesion variant.

Claims

1. A process which comprises making an immunoglobulin heavy chain dimer in which the extracellular domain sequence of a cell surface member of the immunoglobulin gene superfamily, which superfamily member is not a class I or class II major histocompatibility antigen, an immunoglobulin, nor a T-cell receptor α , β , γ or δ chain, is substituted for the variable region of an immunoglobulin heavy chain, which dimer comprises a polypeptide comprising said extracellular domain fused to an immunoglobulin heavy chain constant region, and in which dimer said extracellular domain binds a ligand of said member of the immunoglobulin gene superfamily.
2. A process according to claim 1 wherein the immunoglobulin heavy chain dimer is divalent for said ligand.
3. The process of claim 1 or claim 2 wherein the immunoglobulin sequence is obtained from IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.
4. The process of any preceding claim wherein the superfamily member is CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, an intercellular or neural adhesion molecule, poly-Ig receptor, myelin associated glycoprotein, high affinity IgE receptor, platelet derived growth factor receptor, colony stimulating factor-1 receptor, Fc gamma receptors or the carcinoembryonic antigen.
5. The process of claim 4 wherein the superfamily member is CD2, CD4, CD8, CD28, the γ , δ or ϵ chain of CD3 or the high affinity IgE receptor.
6. The process of claim 5 wherein the superfamily member is CD4.
7. The process of claim 1 wherein the V₁ domain of CD4 or CD4 lacking the transmembrane and cytoplasmic domains is fused at its C-terminus to said constant region.
8. A process which comprises making a polypeptide comprising an extracellular domain of CD4 fused to a polypeptide extraneous to CD4.
9. The process of claim 8 wherein the extraneous polypeptide comprises a signal sequence.
10. The process of claim 8 or claim 9 wherein the extraneous polypeptide is capable of eliciting a humoral immune

response in an animal.

11. The process of claim 10 wherein the extraneous polypeptide is a viral polypeptide or an allergen.
- 5 12. The process of claim 8 wherein the extraneous polypeptide is albumin, apolipoprotein or transferrin.
13. The process of claim 8 wherein the extraneous polypeptide is a cytotoxic polypeptide.
14. A process which comprises making a CD4 polypeptide amino acid sequence variant (a) having a transmembrane domain modified to be incapable of becoming lodged in the cell membrane, or (b) lacking a transmembrane domain.
- 10 15. A process according to claim 14 wherein the transmembrane domain has been modified by substituting an amino acid sequence having a substantially hydrophilic hydropathy profile.
- 15 16. A process which comprises making a composition comprising a CD4 polypeptide amino acid sequence variant (a) having a transmembrane domain modified to be incapable of cell membrane anchorage, or (b) lacking a transmembrane domain.
17. The process of claim 16 wherein the variant comprises a CD4 amino acid sequence capable of binding gp120.
- 20 18. A process which comprises making a CD4 polypeptide variant comprising a fusion protein in which a polypeptide comprising a human CD4 antigen variable (V) region is fused at its C-terminus to the N-terminus of a polypeptide comprising a constant region of an immunoglobulin chain covalently associated with a companion immunoglobulin heavy chain-light chain pair bearing a $V_L V_H$ antibody combining site capable of binding to a predetermined antigen.
- 25 19. The process of claim 18 wherein the CD4 polypeptide variant is $AC_H-(V_L C_L-V_H C_H)$, $(AC_L-AC_H)-(V_L C_L-V_H C_H)$, $(AC_L-V_H C_H)-(V_L C_L-V_H C_H)$, or $(V_L C_L-AC_H)-(V_L C_L-V_H C_H)$, wherein A is a human CD4 antigen V region, V_L and V_H are the variable domains of an immunoglobulin light and heavy chain, respectively, and C_L and C_H are the constant domains of an immunoglobulin light and heavy chain, respectively.
- 30 20. The process of claim 18 or claim 19 wherein the fusion protein comprises the V-J domain of the human CD4 antigen.
21. The process of claim 18 or claim 19 wherein the human CD4 antigen comprises the sequence Asn-Lys-Val-Val-Leu-Gly-Lys-Lys.
- 35 22. The process of any one of claims 18 to 21 wherein the immunoglobulin sequences are from IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.
23. The process of claim 22 wherein the immunoglobulin sequences are from IgG-1 or IgG-3.
- 40 24. The process of claim 22 wherein the immunoglobulin sequences are from IgA or IgM.
25. A process which comprises making nucleic acid encoding a dimer according to any one of claims 1 to 7.
- 45 26. A process which comprises making replicable vector comprising nucleic acid prepared by the process of claim 25.
27. A recombinant host cell comprising nucleic acid prepared by the process of claim 25 or the recombinant vector prepared by the process of claim 26.
- 50 28. A method for preparing a dimer according to any one of claims 1 to 7 comprising recovering the dimer from the culture of a recombinant host cell according to claim 27.
29. A process which comprises making nucleic acid encoding a polypeptide according to any one of claims 8 to 13.
- 55 30. A process which comprises making a replicable vector comprising nucleic acid prepared by the process of claim 29.
31. A recombinant host cell comprising nucleic acid prepared by the process of claim 29 or the recombinant vector prepared by the process of claim 30.

32. A method for preparing a polypeptide according to any one of claims 8 to 13 comprising recovering the polypeptide from the culture of a recombinant host cell according to claim 31.
33. A process which comprises making nucleic acid encoding a polypeptide according to claim 14 or claim 15.
34. A method for preparing a CD4 polypeptide amino acid sequence variant according to claim 14 or claim 15 comprising recovering the variant from the culture of a host cell transfected with the nucleic acid prepared by the process of claim 37.
35. A process which comprises making nucleic acid encoding a CD4 polypeptide variant according to any one of claims 18 to 25.
36. A method of preparing a CD4 polypeptide variant comprising transfecting a host cell expressing nucleic acid encoding an immunoglobulin having a variable region directed against a predetermined antigen, with nucleic acid encoding a fusion protein in which a polypeptide comprising a human CD4 antigen variable (V) region is fused at its C-terminus to the N-terminus of a polypeptide comprising a constant region of an immunoglobulin chain.
37. The method of claim 36 wherein the CD4 polypeptide variant is recovered from the host cell culture.

Patentansprüche

1. Verfahren, umfassend die Herstellung eines Immunglobulin-Schwerketten-Dimers, worin der variable Bereich einer Immunglobulin-Schwerkette durch die Sequenz der extrazellulären Domäne eines Zelloberflächenmitglieds der Immunglobulin-Gen-Überfamilie ersetzt ist, wobei das Überfamilienmitglied kein Haupt-Histokompatibilitäts-Antigen der Klasse I oder Klasse II, kein Immunglobulin und weder die α -, β -, γ - noch die δ -Kette eines T-Zellen-rezeptors ist, wobei das Dimer ein Polypeptid umfaßt, das die extrazelluläre Domäne mit einem konstanten Immunglobulin-Schwerketten-Bereich fusioniert umfaßt, und wobei in diesem Dimer die extrazelluläre Domäne einen Liganden des Mitglieds der Immunglobulin-Gen-Überfamilie bindet.
2. Verfahren nach Anspruch 1, worin das Immunglobulin-Schwerketten-Dimer für diesen Liganden zweiwertig ist.
3. Verfahren nach Anspruch 1 oder Anspruch 2, worin die Immunglobulinsequenz von IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD oder IgM erhalten wird.
4. Verfahren nach einem der vorangegangenen Ansprüche, worin das Überfamilienmitglied CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, ein interzelluläres oder neurales Bindemolekül, Poly-Ig-Rezeptor, mit Myelin assoziiertes Glykoprotein, IgE-Rezeptor mit hoher Affinität, von Blutplättchen stammender Wachstumsfaktorrezeptor, Kolonie stimulierender Faktor-1-Rezeptor, Fc- γ -Rezeptoren oder das carcinoembryonale Antigen ist.
5. Verfahren nach Anspruch 4, worin das Überfamilienmitglied CD2, CD4, CD8, CD28, die γ -, δ - oder ϵ -Kette von CD3 oder der IgE-Rezeptor mit hoher Affinität ist.
6. Verfahren nach Anspruch 5, worin das Überfamilienmitglied CD4 ist.
7. Verfahren nach Anspruch 1, worin die V₁-Domäne von CD4 oder CD4 ohne Transmembran- und zytoplasmatische Domänen an ihrem C-Terminus mit dem konstanten Bereich fusioniert ist.
8. Verfahren, umfassend die Herstellung eines Polypeptids, das eine extrazelluläre Domäne von CD4, fusioniert mit einem zu CD4 fremden Polypeptid, umfaßt.
9. Verfahren nach Anspruch 8, worin das fremde Polypeptid eine Signalsequenz umfaßt.
10. Verfahren nach Anspruch 8 oder 9, worin das fremde Polypeptid bei einem Tier eine humorale Immunreaktion hervorrufen kann.
11. Verfahren nach Anspruch 10, worin das fremde Polypeptid ein virales Polypeptid oder ein Allergen ist.

12. Verfahren nach Anspruch 8, worin das fremde Polypeptid Albumin, Apolipoprotein oder Transferrin ist.
13. Verfahren nach Anspruch 8, worin das fremde Polypeptid ein zytotoxisches Polypeptid ist.
- 5 14. Verfahren, umfassend die Herstellung einer CD4-Polypeptid-Aminosäuresequenzvariante, bei der (a) eine Transmembrandomäne so modifiziert ist, daß sie nicht mehr fähig ist, sich in der Zellmembran anzuordnen, oder der (b) eine Transmembrandomäne fehlt.
- 10 15. Verfahren nach Anspruch 14, worin die Transmembrandomäne durch Substitution einer Aminosäuresequenz mit einem im wesentlichen hydrophilen Hydropathieprofil modifiziert worden ist.
- 15 16. Verfahren, umfassend die Herstellung einer Zusammensetzung, die eine CD4-Polypeptid-Aminosäuresequenzvariante umfaßt, welche (a) eine Transmembrandomäne aufweist, die so modifiziert ist, daß sie nicht mehr zur Verankerung in der Zellmembran fähig ist, oder der (b) eine Transmembrandomäne fehlt.
17. Verfahren nach Anspruch 16, worin die Variante eine CD4-Aminosäuresequenz umfaßt, die zur Bindung von gp120 fähig ist.
- 20 18. Verfahren zur Herstellung einer CD4-Polypeptidvariante, die, ein Fusionsprotein umfaßt, in dem ein Polypeptid, das einen variablen (V-) menschlichen CD4-Antigen-Bereich umfaßt, an seinem C-Terminus mit dem N-Terminus eines Polypeptids fusioniert ist, das einen konstanten Bereich einer Immunglobulinkette umfaßt, die kovalent mit einem begleitenden Immunglobulin-Schwerketten-Leichtketten-Paar assoziiert ist, das eine $V_L V_H$ -Antikörperkombinationsstelle umfaßt, die zur Bindung an ein vorbestimmtes Antigen fähig ist.
- 25 19. Verfahren nach Anspruch 18, worin die CD4-Polypeptidvariante $AC_H(V_L C_L V_H C_H)$, $(AC_L-AC_H)(V_L C_L V_H C_H)$, $(AC_L V_H C_H)(V_L C_L V_H C_H)$ oder $(V_L C_L-AC_H)(V_L C_L V_H C_H)$ ist, worin A ein menschlicher CD4-Antigen-V-Bereich ist, V_L und V_H die variablen Domänen der leichten bzw. schweren Kette eines Immunglobulins sind und C_L und C_H die konstanten Domänen der leichten bzw. schweren Kette eines Immunglobulins sind.
- 30 20. Verfahren nach Anspruch 18 oder 19, worin das Fusionsprotein die V-J-Domäne des menschlichen CD4-Antigens umfaßt.
21. Verfahren nach Anspruch 18 oder 19, worin das menschliche CD4-Antigen die Sequenz Asn-Lys-Val-Val-Leu-Gly-Lys-Lys umfaßt.
- 35 22. Verfahren nach einem der Ansprüche 18 bis 21, worin die Immunglobulinsequenzen von IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD oder IgM stammen.
23. Verfahren nach Anspruch 22, worin die Immunglobulinsequenzen von IgG-1 oder IgG-3 stammen.
- 40 24. Verfahren nach Anspruch 22, worin die Immunglobulinsequenzen von IgA oder IgM stammen.
25. Verfahren zur Herstellung von Nukleinsäure, die für ein Dimer nach einem der Ansprüche 1 bis 7 kodiert.
- 45 26. Verfahren, umfassend die Herstellung eines replizierbaren Vektors, der die nach dem Verfahren nach Anspruch 25 hergestellte Nukleinsäure umfaßt.
27. Rekombinante Wirtszelle, umfassend die nach dem Verfahren nach Anspruch 25 hergestellte Nukleinsäure oder den nach dem Verfahren nach Anspruch 26 hergestellten rekombinanten Vektor.
- 50 28. Verfahren zur Herstellung eines Dimers nach einem der Ansprüche 1 bis 7, umfassend die Gewinnung des Dimers aus der Kultur einer rekombinanten Wirtszelle nach Anspruch 27.
29. Verfahren, umfassend die Herstellung von Nukleinsäure, die für ein Polypeptid nach einem der Ansprüche 8 bis 13 kodiert.
- 55 30. Verfahren, umfassend die Herstellung eines replizierbaren Vektors, der die nach dem Verfahren nach Anspruch 29 hergestellte Nukleinsäure umfaßt.

31. Rekombinante Wirtszelle, die die nach dem Verfahren nach Anspruch 29 hergestellte Nukleinsäure oder den nach dem Verfahren nach Anspruch 30 hergestellten rekombinanten Vektor umfaßt.
32. Verfahren zur Herstellung eines Polypeptids nach einem der Ansprüche 8 bis 13, umfassend die Gewinnung des Polypeptids aus der Kultur einer rekombinanten Wirtszelle nach Anspruch 31.
33. Verfahren, umfassend die Herstellung von Nukleinsäure, die für ein Polypeptid nach Anspruch 14 oder 15 kodiert.
34. Verfahren zur Herstellung einer CD4-Polypeptid-Aminosäuresequenzvariante nach Anspruch 14 oder 15, umfassend das Gewinnen der Variante aus der Kultur einer Wirtszelle, die mit der nach dem Verfahren nach Anspruch 33 hergestellten Nukleinsäure transfiziert ist.
35. Verfahren, umfassend die Herstellung von Nukleinsäure, die für eine CD4-Polypeptid-Variante nach einem der Ansprüche 18 bis 25 kodiert.
36. Verfahren zur Herstellung einer CD4-Polypeptidvariante, umfassend das Transfizieren einer Wirtszelle, die Nukleinsäure exprimiert, welche für ein Immunglobulin mit einem variablen Bereich kodiert, der gegen ein vorbestimmtes Antigen gerichtet ist, mit Nukleinsäure, die für ein Fusionsprotein kodiert, in dem ein Polypeptid, das einen variablen (V-) menschlichen CD4-Antigen-Bereich umfaßt, an seinem C-Terminus mit dem N-Terminus eines Polypeptids fusioniert ist, das einen konstanten Bereich einer Immunglobulinkette umfaßt.
37. Verfahren nach Anspruch 36, worin die CD4-Polypeptid-Variante aus der Wirtszellenkultur gewonnen wird.

Revendications

1. Procédé qui comprend la fabrication d'un dimère chaîne lourde d'immunoglobuline dans lequel la séquence du domaine extracellulaire d'un membre de surface de cellule de la superfamille du gène de l'immunoglobuline, lequel membre de la superfamille n'est pas un antigène d'histocompatibilité majeur de classe I ou de classe II, une immunoglobuline ni une chaîne α , β , γ ou δ d'un récepteur de cellules T, est substituée pour la région variable d'une chaîne lourde d'immunoglobuline, lequel dimère comprend un polypeptide comprenant ledit domaine extracellulaire fusionné à une région constante de chaîne lourde d'immunoglobuline dimère dans lequel ledit domaine extracellulaire lie un ligand dudit membre de la superfamille du gène d'immunoglobuline.
2. Procédé selon la revendication 1, où le dimère chaîne lourde d'immunoglobuline est divalent pour ledit ligand.
3. Procédé selon la revendication 1 ou 2 où la séquence d'immunoglobuline est obtenue à partir de IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD ou IgM.
4. Procédé selon l'une quelconque des revendications précédentes dans lequel le membre de la superfamille est CD1, CD2, CD3, CD4, CD8, CD28, OX-2, Thy-1, une molécule d'adhésion neurale ou intracellulaire, un récepteur poly-Ig, une glycoprotéine associée à une myéline, un récepteur d'IgE à affinité élevée, un récepteur d'un facteur de croissance dérivé des plaquettes, un récepteur du facteur-1 stimulant les colonies, les récepteurs gamma de Fc ou l'antigène carcinoembryonnaire.
5. Procédé selon la revendication 4 où le membre de la superfamille est CD2, CD4, CD8, CD28, la chaîne γ , δ ou ϵ de CD3 ou le récepteur d'IgE à affinité élevée.
6. Procédé selon la revendication 5 où le membre de la superfamille est CD4.
7. Procédé selon la revendication 1 où le domaine V₁ de CD4 ou CD4 manquant des domaines transmembrane et cytoplasmique est fusionné à son terminus C à ladite région constante.
8. Procédé qui comprend la fabrication d'un polypeptide comprenant un domaine extracellulaire de CD4 fusionné à un polypeptide étranger à CD4.
9. Procédé selon la revendication 8 où le polypeptide étranger comprend une séquence signal.

10. Procédé selon la revendication 8 ou la revendication 9 dans lequel le polypeptide étranger est capable d'éliciter une réponse immune humorale chez un animal.
11. Procédé selon la revendication 10 dans lequel le polypeptide étranger est un polypeptide viral ou un allergène.
12. Procédé selon la revendication 8 dans lequel le polypeptide étranger est l'albumine, une apolipoprotéine ou une transferrine.
13. Procédé selon la revendication 8 dans lequel le polypeptide étranger est un polypeptide cytotoxique.
14. Procédé qui comprend la fabrication d'un variant d'une séquence d'acides aminés du polypeptide CD4 (a) ayant un domaine transmembranaire modifié pour être incapable de venir se loger dans la membrane cellulaire, ou (b) manquant d'un domaine transmembranaire.
15. Procédé selon la revendication 14 dans lequel le domaine transmembranaire a été modifié par substitution d'une séquence d'acides aminés ayant un profil d'hydrophatie substantiellement hydrophile.
16. Procédé qui comprend la fabrication d'une composition comprenant un variant d'une séquence d'acides aminés du polypeptide CD4 (a) ayant un domaine transmembranaire modifié pour être incapable d'un ancrage de cellules membranaires, ou (b) manquant d'un domaine transmembranaire.
17. Procédé selon la revendication 16 dans lequel le variant comprend une séquence d'acides aminés de CD4 capable de lier gp120.
18. Procédé qui comprend la fabrication d'un variant d'un polypeptide CD4 comprenant une protéine de fusion où un polypeptide comprenant une région variable (V) d'antigène de CD4 humain est fusionné à son terminus C au terminus N d'un polypeptide comprenant une région constante d'une chaîne d'immunoglobuline associée de façon covalente à une paire chaîne lourde-chaîne légère d'immunoglobuline compagne portant un site de combinaison d'anticorps $V_L V_H$ capable de se lier à un antigène prédéterminé.
19. Procédé selon la revendication 18, dans lequel le polypeptide du variant de CD4 est $AC_H-(V_L C_L-V_H C_H)$, $(AC_L-AC_H)-(V_L C_L-V_H C_H)$, $(AC_L-V_H C_H)-(V_L C_L-V_H C_H)$, ou $(V_L C_L-AC_H)-(V_L C_L-V_H C_H)$, où A est une région V d'antigène de CD4 humain, V_L et V_H sont les domaines variables d'une chaîne légère et lourde d'immunoglobuline, respectivement, et C_L et C_H sont les domaines constants d'une chaîne légère et lourde d'immunoglobuline, respectivement.
20. Procédé selon la revendication 18 ou 19 où la protéine de fusion comprend le domaine V-J de l'antigène CD4 humain.
21. Procédé selon la revendication 18 ou la revendication 19 dans lequel l'antigène CD4 humain comprend la séquence Asn-Lys-Val-Val-Leu-Gly-Lys-Lys.
22. Procédé selon l'une quelconque des revendications 18 à 21 où les séquences d'immunoglobuline proviennent de IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD ou IgM.
23. Procédé selon la revendication 22 où les séquences d'immunoglobuline proviennent de IgG-1 ou IgG-3.
24. Procédé selon la revendication 22 dans lequel les séquences d'immunoglobuline proviennent de IgA ou IgM.
25. Procédé qui comprend la fabrication d'un acide nucléique encodant un dimère selon l'une quelconque des revendications 1 à 7.
26. Procédé qui comprend la fabrication d'un vecteur répliquable comprend un acide nucléique préparé par le procédé selon la revendication 25.
27. Cellule hôte recombinante comprenant un acide nucléique préparé par le procédé de la revendication 25 ou le vecteur recombinant préparé par le procédé de la revendication 26.
28. Méthode de préparation d'un dimère selon l'une quelconque des revendications 1 à 7 comprenant la récupération

du polypeptide de la culture d'une cellule hôte recombinante selon la revendication 27.

29. Procédé qui comprend la fabrication d'un acide nucléique encodant un polypeptide selon l'une quelconque des revendications 8 à 13.

30. Procédé qui comprend la fabrication d'un vecteur répliquable comprenant un acide nucléique préparé par le procédé de la revendication 29.

31. Cellule hôte recombinante comprenant un acide nucléique préparé par le procédé de la revendication 29 ou le vecteur recombinant préparé par le procédé de la revendication 30.

32. Méthode de préparation d'un polypeptide selon l'une quelconque des revendications 8 à 13 comprenant la récupération du polypeptide de la culture d'une cellule hôte recombinante selon la revendication 31.

33. Procédé qui comprend la fabrication d'un acide nucléique encodant un polypeptide selon la revendication 14 ou la revendication 15.

34. Méthode pour préparer un variant d'une séquence d'acides aminés du polypeptide CD4 selon la revendication 14 ou la revendication 15 comprenant la récupération du variant de la culture d'une cellule hôte transfectée avec l'acide nucléique préparé par le procédé de la revendication 37.

35. Procédé qui comprend la fabrication d'un acide nucléique encodant un variant d'un polypeptide CD4 selon l'une quelconque des revendications 18 à 25.

36. Méthode de préparation d'un variant d'un polypeptide CD4 comprenant la transfection d'une cellule hôte exprimant un acide nucléique encodant une immunoglobuline ayant une région variable dirigée contre un antigène prédéterminé, avec l'acide nucléique encodant une protéine de fusion où un polypeptide comprenant une région variable (V) d'antigène de CD4 humain est fusionné à son terminus C au terminus N d'un polypeptide comprenant une région constante d'une chaîne d'immunoglobuline.

37. Méthode selon la revendication 36 dans laquelle le variant du polypeptide CD4 est récupéré de la culture de cellules hôtes.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
84

Fig. 1a

[illegible]

Fig. 1b

```

sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I
avaII      avaII      avaII      avaII      avaII      avaII      avaII      avaII      avaII      avaII
ppuMI      ppuMI      ppuMI      ppuMI      ppuMI      ppuMI      ppuMI      ppuMI      ppuMI      ppuMI
ecoO      ecoO      ecoO      ecoO      ecoO      ecoO      ecoO      ecoO      ecoO      ecoO
avaI      alaNI      alaNI      alaNI      alaNI      alaNI      alaNI      alaNI      alaNI      alaNI
CTCGGGACAGGTCTGCTGGAATCCAAACATCAAGGTTCTGCCACATGGTCCACCCCGAGCTTTAATGCGGTAGCTTATCACAGTTAAATTGGCTAACGCCA
GACCCCTGTCCAGGACGACCTTAGGTGTAGTTCCAAGACGCGGTGTACCCAGGTGGGCTCGAAATACGCCATCAAAATAGTGTCAATTTAACGATTGCGT
350  SerGlyGlnValLeuLeuGluSerAsnIleLysValLeuProThrTrpSerThrProSerPheAsnAlaValValTyrHisSerOC*

nlaIV      nlaIV      nlaIV      nlaIV      nlaIV      nlaIV      nlaIV      nlaIV      nlaIV      nlaIV
bani      bani      bani      bani      bani      bani      bani      bani      bani      bani
hinPI      hinPI      hinPI      hinPI      hinPI      hinPI      hinPI      hinPI      hinPI      hinPI
hhaI      hhaI      hhaI      hhaI      hhaI      hhaI      hhaI      hhaI      hhaI      hhaI
GTCAGGCACCCGTGTATGAAATCTAACAAATGCGCTCATCGTCATCCCTCGGCACCGTCCACCCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCC
CAGTCCGTGGCACATACCTTAGATTGTTACGGGAGTAGCAGTAGGAGCCCGTGGCAGTGGGACCTACGACATCCCGTATCCGGAACCAATACGGGCCATGACGG
1301

mnlI      mnlI      mnlI      mnlI      mnlI      mnlI      mnlI      mnlI      mnlI      mnlI
haeIII     haeIII     haeIII     haeIII     haeIII     haeIII     haeIII     haeIII     haeIII     haeIII
sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I      sau96I
GGGCTCTTGGGGAT      GGGCTCTTGGGGAT      GGGCTCTTGGGGAT      GGGCTCTTGGGGAT      GGGCTCTTGGGGAT      GGGCTCTTGGGGAT
CCCGAGAGACGCCCTA      CCCGAGAGACGCCCTA      CCCGAGAGACGCCCTA      CCCGAGAGACGCCCTA      CCCGAGAGACGCCCTA      CCCGAGAGACGCCCTA
1401

```

Fig. 1c

[illegible]

Fig. 2a

scrFI
 batXI
 aluI
 sacI batNI
 aluI hgiAI
 pvuII bspI286
 ddeI banII
 mboII mnlI
 601 GAGAGCCCCCTGCTAGTACCCCTCAGTGCATGTAGAGTCAAGGGTAAAAACATACAGGGGGGAGAACCTCTCTCCGTCTCAGCTGCTCC
 CTCTCGGGGACCATCATCTGGGGAGTCACTTACATCTCAGGTCCCAATTTTGTATGTCTGGAGGACGACAGTCCACCTCGAGG
 176 GluSerProGlySerSerProSerValGlnCysArgSerProArgGlySerAsnIleGlnGlyLysThrLeuSerValSerGlnLeuGluGln
 scrFI
 batNI
 nlaIV
 mnlI
 701 AGCATAGTGGCACTGACATCCATCTGTTCGACAGACAGAGAGGTGGAGTTCAAATATACATCATCTGCTGTAGCTTTCAGAAAGGCTCCAGCAT
 TCCTATCACCCGTGGAGCTGTAGCTGACAGAACCTGTCTCTTCCACCTCAAGTTTATCTGTAGCACACCAAGTCCGAAAGGTCTCTCCGAGGTGCTA
 210 AspSerGlyThrTrpThrCysThrValLeuGlnAsnGlnLysValGluPheLysIleLeuValValLeuAlaPheGlnLysAlaSerSerIle
 mnlI
 801 AGCTATAGAAAGAGGGGGAACAGGTGGAGTCTCTTCCACCTCCCTTTACAGTTGAAAGCTGACGGGACGTCTCTGCGACGGCGGAG
 TCAGATATCTTCTCCCTGTGTCCACCTCAAGAGAGAGGTGACGGGAATTCAACTTTTCGACTGCCGTACCGCTCGACACCACTCCGCTC
 243 ValTyrLysGluGlyGluGlnValGluPheSerPheProLeuAlaPheThrValGluLysLeuThrGlySerGlyGluLeuTrpTrpGlnAlaGlu
 hphI
 sau3AI
 mnlI
 901 AGGCTCTCTCTCCCAAGTCTTGGATCACCTTGACCTGAGAACAGAGAGTCTCTGTAAACAGGGGTATCCAGGACCTTAAGCTCCAGATGGGCAAGA
 TCCCGAAGGAGGAGTTCAGAACCTTACCTGGAACCTGACCTTCTCTTCCCTTCACAGACATTTTGCCTGATTCGGGATTCGAGTCTTACCCGTTCT
 276 ArgAlaSerSerSerLysSerTrpIleThrPheAspLeuLysAsnLysGluValSerValLysArgValThrGlnAspProLysLeuGlnMetGlyLysLys
 haeIII
 stuI
 haeI
 1001 AGCTCCCTCCACCTTCCCTCCAGGCTTGTCTACATATGCTGCTCTGGAACCTCACCTCGGCTCCCTGACCCCTTAAGCTCCAGATGGGCAAGA
 TCGAGGGCAGGTGGAGTGGAGGGGTCCGGAACGAGTATAGACCGAGACCTTTTCAGTGGGAGCGGGAATTCCTTGTCTTTCAACGTAGT
 310 LeuProLeuHleLeuThrLeuProGlnAlaLeuProGlnLysArgGlyAsnLeuThrLeuAlaLeuGluAlaLysThrGlyLysLeuHleGln
 scrFI
 batNI
 hphI
 mnlI
 1101 GGAAGTGAACCTCTGCTGATGATGAGCCACTCAGCTCCAGAAATTTTACCTGTGAGGTGTGGGACCCACCTCCCTAAGCTGATGCTGAGTTGAA
 CCTTCACTTGGACCACTACTCTCTCGTGTGAGTGTGAGTCTTTTAAACAGGACATCCACACCTCGGTGGGAGGATTCGATCGACCTCAAACTTT
 343 GluValAsnLeuValValMetArgAlaThrGlnLeuGlnLysAsnLeuThrCysGluValTrpIleProThrSerProLysLeuMetLeuSerLeuLys

Fig. 2b


```

          mnli          avai
          ddei          plei
          mstii         scobii         foki         alwNI         ddei         hinfi         alwNI
1201 CTGCAGAACAGGAGGCAAGGTCGAAAGCGGAGAGCGGTGTGGCTGCTGAACCTGAGCGGGGATGTGGCAGTGTCTGTGAGTGACTCGGGAC
          taqi
          mnli
376  GACCTCTGTCTCCTCCGTTCCAGAGCTTCGCCCTCTTCGCCACACCCACGACTTGGGACTCCGCCCTACACCGTACAGACCACTACTGAGCCCTG
          LeuGluAenLySGLuAlaLySValSerLySArgGLuLySAlaValTrpValLeuAenProGluAlaGlyMetTrpGlnCysLeuLeuSerAaspSerGlyGln

          sau96I          alui          msei          nlaIV
          avaiI          avai          nlaII          baai
          ppuMI          hlnfi          scoo          TCCAGGAGCAGCCTTACGTTAGTTCCTCAAGACGGGTGTACAGGTGGGTGCGAATTCGAAATGTGTCAATTTAACGATTGGTCAGTCCGT
1301 AGGTCTGTGCTGGAAATCCAAACATCAAGGTCTTGCACATGTGTCCACCCGAGCTTTAAATCGCGGTGTTATFCAACAGTTAAATTGCTAAACGGCAGTCAGGCA
          TCCAGGAGCAGCCTTACGTTAGTTCCTCAAGACGGGTGTACAGGTGGGTGCGAATTCGAAATGTGTCAATTTAACGATTGGTCAGTCCGT
          ValLeuLeuGluSerAenIleLySValLeuProThrTrpSerThrProSerPheAsuAlaValValTyrHisSerOC*
410

          hinfI          hhaI          foki          pmi          hphi          foki          bstNI
          hinfI          hhaI          foki          pmi          hphi          foki          bstNI
          hinfI          hhaI          foki          pmi          hphi          foki          bstNI
1401 CCGTGTATGAATCTAAGAAATGCGCTCATCTGTCGACCCGTCACTGCTGGATGCTGTAGGCTTAGGCTTGGTTATGCCGGTACTGCCGGGCCCTCT
          GGCACNTACTTAGATTGTACCGGAGTAGCAGTGGAGCCGTGGCAGTGGGACCTACGACATCCGTATCCGAACCAATACGGCCATGACGGCCCGGAGA
          TCGGGGAT
1501 ACGCCCTA

```

Fig. 2c

CD4

Immunoglobulin γ_1 

Soluble rCD4

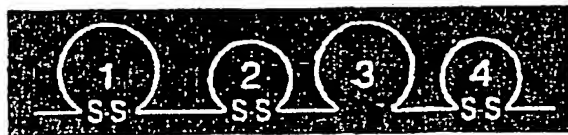
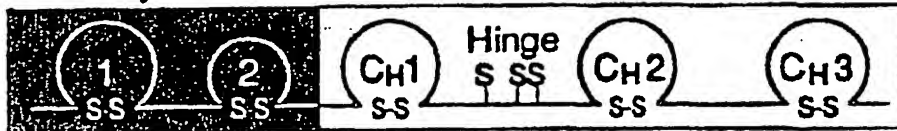
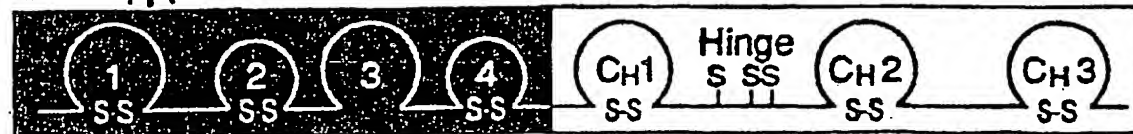
CD4 $_2\gamma_1$ CD4 $_4\gamma_1$ 

Fig. 3

५७

[illegible]

Fig. 4b

sau96I
 avaiI
 nlaIV
 mnlI styI
 ecorI hphI fnu4HI bbvI mboII
 1 GAATTCACCTCACCATCAGCGGCTGCAGCCTGAAGATTTTGCACCTATTACTGCCAACAGTATAAGAGTTTGTGCTCGCTCCTTTCGCGGAGGACCA
 CTTAAGTGAGAGTGCTAGTCGCGGACGTCGGACTTCTAAACCGTGAATTAATGACGGTTCTCATATTCTCAAAACAGCGAGTGAAGCGGCTCCCTGGT
 72 ThrLeuThrIleSerGlyLeuGlnProGluAspPheAlaThrTyrCysGlnGlnTyrLysSerLeuSerLeuThrPheGlyGlyThrLys
 sau3AI fnu4HI mboII mnlI
 101 AGGTGGAGATCAAAACGAACGTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGNAATCTGGAACCTGCCCTCTGTTGTGCCCTGCT
 TCCACCTCTAGTTTGTGTTGACACCGACGCTGGTAGACAGAGTAGAAGGGGGGTGAGACTACTCGTCAACTTAGACCTTGACGGAGACAACACACGGACGA
 104 ValGluIleLysArgThrValAlaAlaProSerValPheIlePheProSerAspGluGlnLeuLysSerGlyThrAlaSerValValCysLeuLeu
 ← V_K $\begin{matrix} \text{TC} \\ \text{CA} \end{matrix}$ haeIII
 xmnI mnlI rsai mnlI
 201 GAATAACTTCTATCCACAGAGAGGCCCCAAAGTACACTGGAAGGTGGATAACGCCCTCCCAATCGGGTAACTCCCAGGAGAGTGTACACAGAGCAGGACAGCAAG
 CTTATTGAAGATAGGGTCTCTCCGGTTTCATGTACCTCCACCTATTGCGGGAGGTTAGCCCTTGAAGGTCTCTCACAGTGTCTCGTCTGTCTGTTTC
 137 AsnAsnPheTyrProArgGluAlaLysValGlnTrpLysValAspAsnAlaLeuGlnSerGlyAsnSerGlnGluSerValThrGluGlnAspSerLys
 sacI hgiAI
 bsp1286
 haeIII
 sau96I aluI
 ecoO banII
 fnu4HI ddeI
 301 GACAGCACCTACAGCCTCAGCAGCACCCCTGACCGCTGAGCAACAGCAGACTACGAGAAACACAAAGTCTACGCCCTGCGAAGTACACCCATCAGGGCCTGAGCT
 CTGTCGTGGATGTCGGAGTCGTCGGAGCTGCGACTGCGACTCGTTCGTCTGATGCTCTTGTGTTTCAGATGCGGACGCTTCACTGGGTAGTCCCGGACTCGA
 170 AspSerThrTyrSerLeuSerSerThrLeuThrLeuSerLysAlaAspTyrGluLysHisLysValTyrAlaCysGluValThrHisGlnGlyLeuSerSer
 aluI mnlI bsp1286 bspMI mnlI ddeI
 401 CGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAGAGGGGAGAGTGTCCCCCACCTGCTCCTCAGT
 CGCGGCAGCTTCTCGAAGTTGTCCCTCTCACAAATCTCCCTTCTCACGGGGGTGGACGAGGAGTCA
 204 ProValThrLysSerPheAsnArgGlyGluCysAM*

Fig. 5